UNIVERSIDAD DE SEVILLA

FACULTAD DE ODONTOLOGÍA

DEPARTAMENTO DE ESTOMATOLOGIA

"EVALUACIÓN DE LA LIBERACIÓN IN VIVO DE IONES METÁLICOS A PARTIR DE APARATOLOGÍA ORTODÓNCICA EN DIVERSAS MATRICES Y SUS POTENCIALES EFECTOS TÓXICOS"

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para optar al título de Doctor por la Universidad de Sevilla con la Mención Internacional

Sevilla, 2015

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ADN: Ácido desoxirribonucleico

AAS: Espectrometría de absorción atómica

Al: Aluminio

ATSDR: Agencia para Sustancias Tóxicas y el Registro de Enfermedades

βTi: Beta Titanio

Cd: Cadmio

col: colaboradores

CONTAM: Panel de Contaminantes en la Cadena Alimentaria de EFSA

Cr: Cromo

CRMs: Materiales de referencia certificados

Cu: Cobre

DL_{50}: Dosis Letal -50

EC: Comisión Europea

EFSA: Autoridad Europea de Seguridad Alimentaria

EGVM: Expertos en Vitaminas y Minerales de Reino Unido

Endo III: Endonucleasa III

ERO: Especies reactivas de oxígeno

Fe: Hierro

g: gramos

FPG: Formamidopirimidina glicosilasa

GFAAS: Espectrometría de absorción atómica horno de grafito

GPx: Glutatión peroxidasa

GSH: Glutatión reducido

HGF: Fibroblastos humanos gingivales

H_2O_2: Peróxido de hidrógeno

HNO_3: Ácido nítrico

IARC: Agencia Internacional para la Investigación sobre el Cáncer

ICP-OES: Espectroscopia de emisión atómica de plasma acoplado

ICP-MS: Espectrometría de Masas con Plasma Acoplado Inductivamente

IDT: Ingesta Diaria Tolerable

IgA: Inmunoglobulina A

i.p.: intraperitoneal

LA: Disolución de ácido láctico y acético, pH 2,5

LOD: Límite de detección

LOQ: Límite de cuantificación

LPO: Peroxidación lipídica

mg: miligramo(s)

MIM brackets: Metal Injection Molding Brackets

mL: mililitro(s)

MN: Micronúcleos

Mn: Manganeso

Mo: Molibdeno

MTT: (3,-[4,5-dimetiltiazol-2-il]2,5difeniltetrazolio bromuro]

NaCl: Cloruro sódico

Ni: Níquel

NiTi: Níquel Titanio

$OH^{\bullet}$: radical hidroxilo

OMS: Organización Mundial de la Salud

p.c.: peso corporal

RFs: Materiales de referencia

SCF: Comité Científico de la Alimentación de la Unión Europea

SRMs: Mmateriales estándar de referencia

SS: Acero inoxidable. Stainless steel

TCA: Ácidos tartárico, citrico y ascórbico, pH 2,2

TMA: Aleaciones Ti-Mo

UL: Nivel de ingesta tolerable máximo (Upper Limit)

µg: microgramo(s) / **µmol:** micromol(es) / **µM**: micromolar

V: Vanadio

Zr: Zirconio

I. RESUMEN / SUMMARY

RESUMEN / SUMMARY

Resumen

La biocompatibilidad de la aparatología ortodóncica es una fuente de investigación en los últimos años ya que está íntimamente relacionada con la liberación de iones metálicos al organismo humano e inducción potencial de efectos biológicos. Dentro del medio intraoral, durante un tratamiento de ortodoncia, que habitualmente comprende 2-3 años, las células orales están en contacto directo con la aparatología ortodóncica. Así mismo, la cavidad oral es un medio ideal para los procesos de corrosión, dadas sus características de humedad, pH y microbiológicas. La aparatología ortodóncica está compuesta por bandas, brackets, arcos y muelles, entre otros dispositivos. A lo largo de los años, los materiales ortodóncicos han evolucionado siguiendo las necesidades de los tratamientos, apareciendo materiales como la aleación níquel-titanio en los años 70, que se caracteriza por un 55% de níquel y 43% de titanio, utilizándose en las primeras fases de tratamiento para el alineamiento y nivelación. Así mismo, aparecieron materiales como el acero, ßTi y TMA (titanio-molibdeno-aluminio).

Con el fin de englobar la literatura ortodóncica en relación a la liberación de metales en la cavidad oral, se realizó una revisión de los estudios más recientes examinando tanto la propia liberación de iones metálicos, como los efectos tóxicos de dichos iones con especial interés en su citotoxicidad y genotoxicidad. Estudios previos sugieren que se debe realizar un análisis de cada caso para tener conciencia del aumento de la variabilidad de los materiales, su composición y los procesos de manufacturación. Los estudios acerca de la toxicidad *in vivo* son escasos, por lo que se deberían realizar nuevas investigaciones para intentar clarificar los resultados contradictorios existentes hasta la actualidad, así como para investigar los mecanismos tóxicos implicados en los efectos observados, con especial énfasis en el daño oxidativo. Por otra parte, son necesarios nuevos estudios de monitorización *in vitro* e *in vivo* que permitan establecer relaciones de causa-efecto entre la liberación de iones metálicos y biomarcadores de

citotoxicidad y genotoxicidad. Los resultados de esta revisión dieron lugar a la siguiente publicación:

IN VITRO AND IN VIVO EVIDENCE OF THE CYTOTOXIC AND GENOTOXIC EFFECTS OF METAL IONS RELEASED BY ORTHODONTIC APPLIANCES: A REVIEW *(Martín-Cameán A y col., 2015. Enviado a Environmental Toxicology and Pharmacology, en revisión).*

Además, se ha llevado a cabo una revisión sistemática siguiendo las directrices PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). Empleando tanto fuentes electrónicas [CENTRAL,MedLine;SCOPUS; EMBASE, Cochrane Library, ISI Web of Science, PASCAL, OVID HealthSTAR, y EBM] y búsquedas manuales [OpenGrey; Google Scholar] se analizaron los efectos cito/genotóxicos de este tipo de aplicaciones ortodoncias en humanos. Se seleccionaron 17 artículos y 6 estudios finalmente cumplieron los criterios (PICOS). Se observaron diferencias significativas en la mayoría de los estudios (5 de 6) respecto a daños citotóxicos y genotóxicos después de un tratamiento corto (1-3 meses) y a mas largo plazo (24-48 meses). Algunos estudios que evaluaron efectos post-tratamiento (2 de 3) no encontraron diferencias significativas con respecto a los controles después de eliminar las aplicaciones ortodóncicas. Se concluye por tanto, la necesidad de llevar a cabo rigurosos ensayos clinicos aleatorios para determinar la continuidad de los daños cito/genotóxicos inducidos durante el tratamiento ortodóncico en población joven (12-26 años). Los resultados de esta revisión dieron lugar a la siguiente publicación:

GENOTOXIC, CYTOTOXIC EFFECTS AND GENE EXPRESSION CHANGES INDUCED BY ORTHODONTIC FIXED APPLIANCES OVER ORAL MUCOSA CELLS: A SYSTEMATIC REVIEW. *Martín-Cameán A et al., 2015. Submitted to Dental Materials (under revision)*

Diversos estudios han evaluado la liberación de iones metálicos a partir de aparatología ortodóncica en fluidos biológicos. La liberación de los elementos se ha medido principalmente mediante espectrometría de absorción atómica (AAS),

espectrometría de emisión atómica con plasma acoplado inductivamente (ICP-AES) o a través de espectrometría de masas con plasma acoplado inductivamente (ICP-MS). La mayoría de ellos han concluido que no se alcanzan concentraciones tóxicas en saliva y suero. Sin embargo, concentraciones no tóxicas pueden ser suficientes para producir cambios biológicos en la mucosa oral.

Debido a que la liberación de metales en células de mucosa oral, que han estado en contacto prolongado con la aparatología fija, en pacientes de ortodoncia se ha estudiado mínimamente y no existen datos de validación previa disponibles, consideramos de importancia desarrollar y optimizar un procedimiento analítico para determinar titanio (Ti), vanadio (V), zirconio (Zr), cobalto (Co), cromo (Cr), cobre (Cu) y níquel (Ni) en células de mucosa oral en pacientes con y sin tratamiento de ortodoncia mediante ICP-MS. El procedimiento analítico se basa en la extracción y digestión de las muestras en medio ácido y cuantificación simultánea de los elementos. El método fue validado adecuadamente: la ecuación de regresión fue calculada a partir de los estándares preparados en la misma matriz sin células de mucosa oral y el rango lineal fue de 0.5-50.0 ng/mL para el Zr, de 5.0-50.0 ng/mL para Ti y V, y de 2.0-100.0 ng/mL para Co, Cr, Cu y Ni. Los límites de detección fueron de 0.9, 2.8 y 0.4 ng/mL para Ti, V y Zr, respectivamente; mientras que fueron de 0.10, 0.38, 0.49 y 0.67 ng/mL para Co, Cr, Cu y Ni, respectivamente. Los límites de cuantificación fueron de 1.8, 3.4 y 0.7 ng/mL para Ti, V y Zr, respectivamente, mientras que fueron de 0.20, 1.13, 0.98 y 1.81 ng/mL para Co, Cr, Cu y Ni, respectivamente. Los porcentajes de recuperación (%) obtenidos oscilaron entre 101-108 para el Ti, 98-111 para el V, 92-104 para el Zr, 104-109 para el Co, 103-107 para el Cr, 106-113 para el Cu y 84-110 para el Ni. Los datos de precisión intermedia (RSD%) fueron adecuados para todos los elementos, a los tres niveles de concentración elegidos. El método resultó robusto para los tres factores que se consideraron en el proceso de tratamiento de la muestra: tiempo de calentamiento, volumen de agua desionizada, y volumen de HNO_3 PlasmaPure 65% utilizado para diluir las muestras, lo cual

permite su validación y aplicación a células de mucosa oral en pacientes ortodóncicos. Los métodos validados se aplicaron con éxito a la determinación de la liberación de dichos iones metálicos en 40 pacientes, 20 de los cuales se encontraban bajo tratamiento de ortodoncia (13-15 meses) y 20 individuos control. Se evaluaron los resultados obtenidos, encontrándose contenidos significativamente superiores de Co, Cr , Cu y Ni en el grupo tratado en comparación con el grupo control, siendo las concentraciones detectadas inferiores a las Ingestas diarias tolerables (IDT) (Ni, Co) o a sus niveles máximos de ingesta tolerable establecidos (UL) de los elementos estudiados (Cr, Cu). Los resultados de estos experimentos han dado lugar a las siguientes publicaciones:

VALIDATION OF A METHOD TO QUANTIFY TITANIUM, VANADIUM AND ZIRCONIUM IN ORAL MUCOSA CELLS BY INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (ICP-MS) *(Martín Cameán A y col., 2014. Talanta 118:238-244)*

DEVELOPMENT AND VALIDATION OF AN INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS) METHOD FOR THE DETERMINATION OF COBALT, CHROMIUM, COPPER AND NICKEL IN ORAL MUCOSA CELLS *(Martín-Cameán A y col., 2014. Microchemical Journal 114:73-79)*

La cavidad oral es un medio particularmente ideal para la biodegradación de metales debido a sus propiedades iónicas, térmicas, microbiológicas y enzimáticas. En este contexto, las aleaciones ortodóncicas emiten corrientes electrogalvánicas tomando como medio la saliva, produciendo una liberación de iones metálicos a la mucosa oral. Diversos factores afectan a la liberación de iones, tales como el proceso de manufacturación, tipo de aleación, características de la superficie del material y envejecimiento de la aleación.

Los metales no son biodegradables, y su liberación constante puede producir efectos tóxicos irreversibles debido a su acumulación en los tejidos. El pelo de cuero cabelludo humano es un vehículo de excreción de sustancias diversas (metales pesados, drogas de adicción etc.,), llegando a estar considerada esta matriz no invasiva como uno de los materiales biológicos más importantes de

monitorización ambiental. A pesar de las numerosas ventajas que presenta el pelo en biomonitorización humana, los procedimientos analíticos para la determinación de liberación de metales en ortodoncia en dicha matriz son escasos, existiendo únicamente un estudio preliminar (procedimiento no validado, muy escaso número de pacientes) que demostró la no existencia de diferencias significativas en el contenido de algunos elementos metálicos en pelo de pacientes bajo tratamiento.

Nuestro estudio se centró en la evaluación de niveles de Cu, Cr, Fe, Mn y Ni en pelo humano de una amplia población tratada con aparatología ortodóncica (n=70) para determinar, si la concentración de un metal dado estaba influenciado por el tratamiento ortodóncico en comparación a un grupo control (n=56). Los niveles de los compuestos metálicos se determinaron a través de espectrometría de absorción atómica (AAS), con diferentes modalidades, llama (Cu, Fe) y cámara de grafito (GF-AAS) (Cr, Mn, Ni). Se estudió la influencia de factores individuales (género, edad) en las concentraciones de metales, se estudiaron interacciones interelementales mediante la evaluación de coeficientes de correlación entre elementos, así como mediante un análisis de regresión múltiple. Las diferencias en el contenido metálico en pelo fue significativamente mayor sólo en el caso de Mn cuando se comparó con el grupo control, aunque los contenidos pueden considerarse de la misma magnitud que otras poblaciones control y, consecuentemente, no se asocian a riesgos debidos al tratamiento. El tratamiento de ortodoncia incrementó significativamente los niveles de Mn en pacientes jóvenes (<20 años) cuando se compararon con el grupo control. El análisis del pelo humano es un buen método para investigar la liberación de elementos a partir de la aparatología ortodóncica. Los resultados de este experimento dieron lugar a la siguiente publicación:

BIOMONITORIZATION OF CHROMIUM, COPPER, IRON, MANGANESE AND NICKEL IN SCALP HAIR FROM ORTHODONTIC PATIENTS BY ATOMIC ABSORPTION SPECTROMETRY *(Martín-Cameán A y col., 2014. Environmental Toxicology and Pharmacology 37:759-771)*

Los microtornillos se utilizan, a día de hoy, habitualmente en los tratamientos de ortodoncia gracias a sus buenos resultados en la práctica clínica. Estos aditamentos se han popularizado ampliamente debido a su simplicidad en el manejo, bajo coste y la mínima necesidad de colaboración por parte del paciente. A pesar del elevado uso de los microtornillos en ortodoncia recientemente, los datos en la literatura ortodóncica en relación a su biocompatibilidad son muy escasos. Algunos metales propios de su composición como Ni y Cr pueden causar hipersensibilidad, dermatitis, asma y citotoxicidad. Así mismo, tienen un potencial genotóxico y carcinogénico significativo, efectos no relacionados con la dosis de exposición.

La evaluación de los agentes genotóxicos se puede llevar a cabo mediante el análisis del daño de ADN primario, como se realiza en el ensayo cometa o "alkaline single cell gel electrophoresis". Este ensayo es el método de elección para la medición de daño de ADN en células humanas en poblaciones expuestas a agentes genotóxicos. Hasta ahora, no existían estudios acerca del potencial genotóxico de los microtornillos en células de mucosa oral en humanos. Teniendo en cuenta la ausencia de estudios en este campo, el objetivo de nuestro estudio fue investigar el daño de ADN en células bucales de pacientes en tratamiento de ortodoncia mediante el ensayo del cometa en comparación con pacientes en tratamiento ortodóncico con microtornillos, y con respecto a un grupo control no tratado y un grupo de personas fumadoras (grupo control positivo). En el grupo de pacientes con tratamiento ortodóncico y en el de ortodoncia+microtornillos se obtuvo un incremento significativo (2 veces más) de %ADN en la cola del cometa en comparación con el grupo control. Las mujeres mostraron un aumento significativo del %ADN en todos los tratamientos en comparación con el grupo control, mientras que los hombres mostraron daño de forma significativa únicamente en el grupo de ortodoncia y microtornillo. En conclusión, la aparatología ortodóncica convencional induce genotoxicidad, y la incorporación

de los microtornillos estudiados no implica un aumento adicional de daño del ADN. Los resultados de este experimento dieron lugar a la siguiente publicación:

EVALUATION OF GENOTOXICITY OF ORTHODONTIC MINISCREWS ON MUCOSA ORAL CELLS BY THE ALKALINE COMET ASSAY *(Martín-Cameán A y col., 2014. Toxicology Mechanisms and Methods, aceptado abril 2015)*

Tras confirmar el efecto genotóxico de la aparatología ortodóncica y la inocuidad de los microtornillos en cuanto al daño celular adicional, quisimos cuantificar las diferencias en la liberación de metales como el aluminio (Al), cobre (Cu), cromo (Cr), manganeso (Mn), níquel (Ni), titanio (Ti) y vanadio (V) en células orales de pacientes bajo tratamiento ortodóncico convencional (brackets, bandas y arcos) en comparación con pacientes tratados adicionalmente con microtornillos, y con, respecto a un grupo control, utilizando ICP-MS. Las resultados obtenidos revelaron el siguiente orden ascendente: Cr < Ni < Ti < Cu < Al, y el Co y V fueron prácticamente no detectados. Se encontraron diferencias significativas en comparación con el grupo control para el Cu en el grupo ortodóncico, y para el Ni en ambos grupos, tanto grupo ortodóncico como grupo ortodoncia+microtornillo. Se realizaron correlaciones potenciales entre los elementos metálicos, encontrándose una correlación positiva Al/Ti, y en relación a varios factores clínicos. Se concluye que los microtornillos no incrementan de forma significativa la liberación de metales. Los resultados de este experimento dieron lugar a la siguiente publicación:

IN VIVO DETERMINATION OF ALUMINIUM, COBALT, CHROMIUM, COPPER, NICKEL, TITANIUM AND VANADIUM IN ORAL MUCOSA CELLS FROM ORTHODONTIC PATIENTS WITH MINI-IMPLANTS BY INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (ICP-MS) *(Martín-Cameán A y col., 2015. Enviado a Journal of Trace Elements in Medicine and Biology, en revisión)*

Para finalizar, teniendo en cuenta los resultados derivados de los experimentos realizados en la presente Tesis Doctoral, queda demostrada la liberación de iones metálicos a partir de aparatología ortodóncica, incluyendo

microtornillos, a partir de pelo y células de mucosa oral, y su componente genotóxico, contribuyendo de esta forma a ampliar el conocimiento que actualmente podemos encontrar en la bibliografía científica en relación a estos materiales.

Summary

The biocompatibility of orthodontic appliances has been worth of research in the last years as it is closely related with the release of metallic ions in the human body and the induction of potential biologic effects. Inside the intraoral environment, during an orthodontic treatment, that usually takes 2-3 years, oral cells are in direct contact with the orthodontic appliance. Also, the oral cavity is an ideal place for corrosion processes, taking into account its characteristics such as humidity, pH and microbiology. Orthodontic appliances are composed by bands, brackets, arches and springs, among other devices. Along the years, orthodontic materials have evolved following the treatment needs, with the appearance of materials such as nickel-titanium alloy in the 70s, which contains 55% Ni and 43% Ti in its composition. This is used in the early stage of the treatment, for the alignment and leveling. Moreover, other materials have appeared, such as steel, ßTi and TMA (titanium-molybdenum-aluminum).

With the aim of compiling the scientific orthodontic literature in relation to the release of metallic ions in the oral cavity, a review including the most recent studies dealing with metallic ions release and their toxic effects, with a special focus on cytotoxicity and genotoxicity, was performed. Previous studies suggest that a case by case evaluation is required, taking into account the increase of material's variability, their composition and the different manufacturing processes. Also, *in vivo* toxicity studies are scarce. Therefore, further research should be performed to try to clarify the contradictory results available nowadays, as well as to elucidate the toxic mechanisms involved in the observed effects, particularly oxidative stress. On the other hand, *in vitro* and *in vivo* monitoring studies are required to establish cause-effect relationships between the release of metallic ions and cytotoxicity and genotoxicity biomarkers. The results obtained led to the following publication:

IN VITRO AND IN VIVO EVIDENCE OF THE CYTOTOXIC AND GENOTOXIC EFFECTS OF METAL IONS RELEASED BY ORTHODONTIC APPLIANCES: A REVIEW *(Martín-Cameán A et al., 2015. Submitted to Environmental Toxicology and Pharmacology, under revision)*
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Moreover, a systematic review has been performed in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Electronic [CENTRAL,MedLine;SCOPUS; EMBASE, Cochrane Library, ISI Web of Science, PASCAL, OVID HealthSTAR, and EBM] and manual searches [OpenGrey; Google Scholar] were employed to analyze the genotoxic/cytotoxic effects of these types of oral appliances in humans. From the initial electronic search (27902), 17 articles were retrieved and 6 studies [low risk of bias(LRB)] finally met the eligibility criteria [PICOS]. Significant differences were observed by most of the studies (5 out of 6) regarding a critically acute detectable geno and cytotoxic effects after appliance using, in the short (at 1 and 3 months) and long term (24-48 months) evaluation. Nevertheless, some of the studies evaluating post-removable effects (2 out of 3) conclude that this effects at the DNA or cellular level were not statistically significant different to controls after removing the oral aggression. In conclusion, despite no further detection of these effects is described by a few studies after removing the appliances, additional rigorous randomized clinical trials are needed to explore to what extent no acquired damage is observed in the oral mucosa in the young target population (12-26 years old). The results obtained led to the following publication

GENOTOXIC, CYTOTOXIC EFFECTS AND GENE EXPRESSION CHANGES INDUCED BY ORTHODONTIC FIXED APPLIANCES OVER ORAL MUCOSA CELLS: A SYSTEMATIC REVIEW. *Martín-Cameán A et al., 2015. Submitted to Dental Materials (under revision)*

Different studies have evaluated the release of metallic ions from orthodontic appliances in biologic fluids. The release of elements has been measured mainly by Atomic Absorption Spectrometry (AAS), Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) or Inductively

Coupled Plasma Mass Spectrometry (ICP-MS). Most of them concluded that toxic concentrations are not reached in saliva and serum. However, non toxic concentrations can be enough to induce biologic effects in the oral mucosa.

Due to the scarce research on the release of metals in oral mucosa cells, in direct contact with fixed orthodontic appliances in orthodontic patients, and the lack of validation data available, we considered important to develop and optimize an analytical procedure to determine titanium (Ti), vanadium (V), zirconium (Zr), cobalt (Co), chromium (Cr), copper (Cu) and nickel (Ni) in oral mucosa cells from patients with and without orthodontic treatment by ICP-MS. The analytical procedure is based on the extraction and digestion of the samples in acid conditions and quantification of the elements simultaneously. The method was properly validated: the regression equation was calculated from standards prepared with the same matrix without oral mucosa cells and the linear range was 0.5-50.0 ng/mL for Zr, 5.0-50.0 ng/mL for Ti and V, and 2.0-100.0 ng/mL for Co, Cr, Cu and Ni. Detection limits were 0.9, 2.8 and 0.4 ng/mL for Ti, V and Zr, respectively, and 0.10, 0.38, 0.49 and 0.67 ng/mL for Co, Cr, Cu and Ni, respectively. Quantification limits were 1.8, 3.4 and 0.7 ng/mL for Ti, V y Zr, respectively, and 0.20, 1.13, 0.98 and 1.81 ng/mL for Co, Cr, Cu y Ni, respectively. Recovery percentages (%) obtained ranged between 101-108 for Ti, 98-111 for V, 92-104 for Zr, 104-109 for Co, 103-107 for Cr, 106-113 for Cu and 84-110 for Ni. Intermediate precision data (RSD%) were adequate for all the elements at the three concentration levels selected. The present method showed to be robust for the three factors considered: heating time, volume of the deionized water, and volume of PlasmaPure 65% HNO_3 used to dilute the samples, which permits its validation and application to oral mucosa cells from orthodontic patients. The validated methods were successfully applied to the determination of the above mentioned metallic ions in 40 patients, 20 of them under an orthodontic treatment (13-15 months) and 20 control subjects. Results obtained were evaluated and significant higher levels of Co, Cr, Cu and Ni were found in the

orthodontic group in comparison to the control group. The concentrations detected were lower than the Tolerable Daily Intake (TDI) (Ni, Co) or to the Tolerable Upper Intake Levels (UL) established for the selected elements (Cr, Cu). The results of these experiments led to the following publications:

VALIDATION OF A METHOD TO QUANTIFY TITANIUM, VANADIUM AND ZIRCONIUM IN ORAL MUCOSA CELLS BY INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (ICP-MS) *(Martín Cameán A et al., 2014. Talanta 118:238-244)*

DEVELOPMENT AND VALIDATION OF AN INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS) METHOD FOR THE DETERMINATION OF COBALT, CHROMIUM, COPPER AND NICKEL IN ORAL MUCOSA CELLS *(Martín-Cameán A et al., 2014. Microchemical Journal 114:73-79)*

The oral cavity is particularly an ideal environment for the biodegradation of metals due to its ionic, thermal, microbiologic and enzymatic properties. In this context, the orthodontic alloys emit electrogalvanic currents with saliva, and consequently, release metal ions to the oral mucosa. Different factors influence the ionic release, such as the manufacturing process, type of alloy, superficial characteristics of the material and the alloy ageing.

Metals are not biodegradable substances and their constant release can lead to irreversible toxic effects due to their accumulation into tissues. The human scalp hair is an excretion vehicle for different compounds (heavy metals, drugs of abuse, etc.) and this non invasive matrix is considered as one of the most important biologic materials for environmental monitoring. In spite of the high number of advantages that the hair has for human biomonitoring purposes, the analytical procedures for the determination of metal release from orthodontic appliances in this matrix are scarce. To the extent of our knowledge there is only one preliminary study (not validated, with a short number of patients) that showed that there were no significant differences in the content of some metallic elements in hair from patients with orthodontic treatment.

Our study focused on the evaluation of Cu, Cr, Fe, Mn and Ni contents in the scalp hair of a wide population under orthodontic treatment (n=70) to determine whether the concentration of a particular metal was influenced by the orthodontic treatment in comparison to the congrol group (n=56). Metallic ions contents were determined by Atomic Absorption Spectrometry (AAS) with different variants, flame (Cu, Fe) and graphite furnace (GF-AAS) (Cr, Mn, Ni). The influence of individual factors (sex, age) on the metal concentrations was studied. Also, interelemental interactions were studied by evaluation of the correlation coefficients between elements, and by a multiple regression analysis. Differences in the metallic content in scalp hair were only significantly higher for Mn in comparison to the control group, but its contents were similar to those found in other control populations, and therefore they are not associated to the treatment. The orthodontic treatment increased significantly Mn content in young patients (<20 years) in comparison to the control group. The analysis of scalp hair is a good method to investigate the release of elements from orthodontic appliances. The results obtained in this experiment were published in the following manuscript:

BIOMONITORIZATION OF CHROMIUM, COPPER, IRON, MANGANESE AND NICKEL IN SCALP HAIR FROM ORTHODONTIC PATIENTS BY ATOMIC ABSORPTION SPECTROMETRY *(Martín-Cameán A et al., 2014. Environmental Toxicology and Pharmacology 37:759-771)*

Miniscrews are frequently used nowadays in orthodontic treatments due to their good results in the clinical practice. The use of these devices has been widely extended due to their simple use, low cost and almost no need of the patient cooperation. In spite of their wide use in the last years, data in the orthodontic literature in relation to their biocompatibility are very scarce. Some characteristic metals of their composition, such as Ni and Cr can cause hypersensitivity, dermatitis, asthma and cytotoxicity. Also, they have a significant genotoxic and carcinogenic potential, effects that are not related with the dose of exposure.

The evaluation of genotoxic agents can be performed by the analysis of primary DNA damage, such as the application of the comet assay or "alkaline single cell gel electrophoresis". This assay is the selected method for detecting DNA damage in human cells in populations exposed to genotoxic agents. Up to now, there were no studies regarding the genotoxic potential of miniscrews in human oral mucosa cells. Taking into account the lack of studies in this field, the aim of our study was to investigate the DNA damage in buccal cells from orthodontic patients by using the comet assay, in comparison to patients with orthodontic appliances and miniscrews and in comparison with a control (non treated) group and a group of smokers (positive control group). The orthodontic and the orthodontic + miniscrew groups showed a significant and similar increase (2-fold) of the %DNA in tail in comparison to the control group. Women showed a significant increase in the % DNA in all treatments in comparison to the control group, whereas men showed significant changes only in the orthodontic + miniscrew group. In conclusion, conventional orthodontic appliances induced genotoxicity and the incorporation of the miniscrews selected to the treatment does not result in a higher increase of DNA damage. The results of this experiment were published in the following manuscript:

EVALUATION OF GENOTOXICITY OF ORTHODONTIC MINISCREWS ON MUCOSA ORAL CELLS BY THE ALKALINE COMET ASSAY *(Martín-Cameán A et al., 2014. Submitted to Toxicology Mechanisms and Methods, under revision).*

Once the genotoxic effect of orthodontic appliances was confirmed and also that miniscrews did not increase this damage, we aimed to quantify the differences in the released content of metals such as aluminum (Al), copper (Cu), chromium (Cr), manganese (Mn), nickel (Ni), titanium (Ti) and vanadium (V) in oral cells from patients with a traditional orthodontic treatment (brackets, bands and arches) in comparison to patients treated additionally with miniscrews, and in comparison to a control group, by ICP-MS. The results obtained showed the following increasing trend: Cr < Ni < Ti < Cu < Al, and Co and V were

practically not detected. Significant differences were found in comparison with the control group for Cu in the orthodontic group and for Ni in both groups, the orthodontic group and the orthodontic + miniscrew group. Potential correlations among the metallic elements were investigated and a positive correlation Al/Ti was found, as well as in relation to different clinical factors. It is considered that miniscrews do not increase significantly metal release. The results obtained in this experiment led to the following publication:

IN VIVO DETERMINATION OF ALUMINIUM, COBALT, CHROMIUM, COPPER, NICKEL, TITANIUM AND VANADIUM IN ORAL MUCOSA CELLS FROM ORTHODONTIC PATIENTS WITH MINI-IMPLANTS BY INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (ICP-MS) *(Martín-Cameán A et al., 2015. Submitted to Journal of Trace Elements in Medicine and Biology, under revision)*

Finally, taking into account the results derived from the experiments performed in the present Doctoral Thesis, it has been proved the release of metallic ions from orthodontic appliances, including miniscrews, from scalp hair and oral mucosa cells, as well as their genotoxic effects. These results have contributed to increase the scientific knowledge available in the orthodontic literature about these materials.

II. INTRODUCCIÓN / INTRODUCTION

1. APAROTOLOGIA ORTODÓNCICA: ASPECTOS ENERALES

La aparatología ortodóncica fija intraoral incluye brackets, bandas, y arcos, que están realizados con aleaciones conteniendo níquel (Ni), cromo (Cr) en diferentes porcentajes, así como manganeso (Mn), hierro (Fe) y cobre (Cu) (Iijima y col., 2006, Regis y col., 2011).

Las aleaciones compuestas de Ni están presentes en un número abundante y gran variedad de aparatos, auxiliares y utensilios ortodóncicos, llegando a convertirse, por ello, en parte integral de la mayoría de las intervenciones diarias ortodóncicas. En la Tabla 1, observamos las aplicaciones que poseen Ni, de las cuales una gran parte pertenece a aleaciones de acero inoxidable tanto en arcos como en brackets.

Categoría	Material
Aparatología estándar	Brackets Bandas
Utensilios de tratamiento	Arcos de acero inoxidable Arcos de Níquel-Titanio (NiTi) Arcos de CoCrNi (Elgiloy)
Auxiliares mecánicos	Arco lingual Barra transpalatina
Auxiliares misceláneos	Ligaduras de acero inoxidable Hooks Kobayashi Coil springs
Aparatología fija de expansión	Quad-helix, Disyuntores Anclaje extraoral Minitornillos
Aparatología removible	Componentes de acero inoxidable de la placa de Hawley y variaciones
Intervenciones terapéuticas complejas	Placas y tornillos de cirugía ortognática Aparatos de distracción osteogénica

Tabla 1. Material ortodóncico con Ni en su composición (Eliades y col., 2002).

Andreasen y Hilleman (1971) introdujeron los arcos de níquel-titanio (NiTi) en ortodoncia a principios de 1970. Esta aleación se caracteriza por contener un 55% de Ni y 43% de Ti (Laino y col., 2012). Las aleaciones de NiTi se utilizan diariamente, especialmente en la fase de alineamiento y nivelación al principio del tratamiento de ortodoncia, gracias a sus óptimas propiedades mecánicas (Petoumeno y col., 2009). La pseudoelasticidad de los arcos de NiTi nos permite la aplicación de fuerzas ligeras de forma continua con activaciones prolongadas que dan lugar a la disminución de traumas tisulares y menor incomodidad del paciente, facilitando el movimiento dentario (Gil y col., 1998). Goldberg y Burston (Goldberg y Burston, 1979) subrayaron que es posible crear un arco ortodóncico con propiedades elásticas interesantes procesando 11% molibdeno (Mo), 6% zirconio (Zr), y 5% beta titanium, conteniendo vanadio (V). Con ello, apareció la aleación multifuncional "Gum metal".

Las aleaciones de NiTi combinan el efecto memoria de forma y la súper-elasticidad con unas excelentes propiedades mecánicas y de corrosión, así como gran nivel de biocompatibilidad. A pesar de estas ventajas, la ausencia de un coeficiente de baja fricción hace difícil el uso óptimo de estos materiales en aparatología ortodóncica (Gil y col., 1998).

Las aparatologías ortodóncicas compuestas de aleaciones de Ni son únicas en cuanto al hecho de que no se implantan en el interior de un tejido, sino que se colocan en una cavidad abierta. A diferencia de los materiales implantados, los ortodóncicos poseen un continuo patrón de reacción con los factores medioambientales presentes en la cavidad oral.

Con el fin de determinar el factor óptimo de seguridad de los materiales ortodóncicos, se deben considerar las variaciones de las propiedades mecánicas y su deterioro, como la fatiga por estrés continuo en un doblez durante el movimiento dentario y la corrosión en el medio oral.

En 1997, en Japón, Kanomi utilizó los mini-implantes para anclaje de movimientos ortodóncicos. La utilización de minitornillos en ortodoncia para

mejorar el anclaje ha evolucionado en los últimos años, con numerosas aplicaciones incluyendo la retracción de dientes anteriores, corrección de mordidas abiertas, distalamiento e intrusión dentaria. Los dispositivos de anclaje temporal recientes se pueden clasificar en: biocompatibles o biológicos en la naturaleza. Ambos grupos se pueden subclasificar en función del mecanismo de unión al hueso, bioquímico (osteointegrado) o mecánico (estabilizado corticalmente). Las características ideales de los materiales de la aparatología ortodóncica son:

· No tóxico.
· Biocompatible.
· Excelentes propiedades mecánicas.
· Resistencia al estrés y tensión.
· Resistencia a la corrosión.

En un primer momento, el material de elección para la fabricación de microtornillos fue Titanio puro comercial (cp Ti), pero para mejorar sus propiedades mecánicas se han incorporado algunos elementos como el aluminio (Al), vanadio (V) y el hierro (Fe) (Carvalho y Tarkany, 2014). Los microtornillos se han popularizado extensivamente debido a su simplicidad en el manejo, bajo coste y la mínima necesidad de colaboración del paciente (Papadopoulos y Tarawneh, 2007; Papageorgiou y col., 2012). Aunque el desarrollo de los dispositivos de anclaje temporal o microtornillos es satisfactorio, su biocompatibilidad es un criterio importante que debe ser comprobado tanto en un modelo animal como humano (Malkoc y col. 2012). A pesar del alto incremento en la utilización de este tipo de dispositivos en los últimos años, los datos en la literatura ortodóncica acerca de la biocompatibilidad de los microtornillos son muy escasos (Morais y col., 2007, De Morais y col., 2009; Malkoc y col., 2012).

2. CORROSIÓN. RESISTENCIA A LA CORROSIÓN

El término “Corrosión” lo podemos definir como el proceso de interacción entre un material sólido y el medio químico en el que se encuentra, dando lugar a

la pérdida de substancia del material, cambios de sus características estructurales o pérdida de su integridad estructural (Chaturvedi, 2008 http://orthocj.com/journal/uploads/2008/01/0054_en.pdf).

La corrosión de una aleación ocurre cuando elementos del mismo se ionizan, esto es, elementos que inicialmente no están cargados en su interior, pierden electrones, se cargan positivamente y son liberados a la disolución. Desde el punto de vista de la biocompatibilidad, la corrosión de una aleación indica que es capaz de afectar a los tejidos de su alrededor. La liberación de elementos puede o no causar problemas en los tejidos (Wataha, 2000). La corrosión se puede medir de varias formas (Wataha y Schmalz, 2009):

- Observando el deterioro o alteraciones del color de su superficie (ej. tinciones).
- Observando el material en relación con alteraciones del flujo de corriente, mediante ensayos electroquímicos.
- Midiendo directamente la liberación de elementos por diferentes técnicas analíticas.

Quizás la medida más relevante de corrosión desde el punto de vista de la biocompatibilidad es la identificación y cuantificación de los elementos liberados (Arenhohlt-Bindslev y col., 2009).

En el medio intraoral se produce corrosión de las aleaciones ortodóncicas, independientemente de la estructura metalúrgica de la aleación. En dicho medio intraoral son posibles numerosos tipos de corrosiones electroquímicas, ya que la saliva es un electrolito débil (Chaturvedi, 2008). Dentro de la cavidad oral, durante un tratamiento de ortodoncia, las células orales están en íntimo contacto con la aparatología metálica. Cada tratamiento de ortodoncia tiene una duración media de entre 24-30 meses y, durante este tiempo, los procesos de corrosión están normalmente presentes (Amini col., 2008). La asociación de diferentes metales en el medio intraoral, donde la saliva es el medio de conexión, da lugar a corrientes electrogalvánicas que producen una descarga de iones y compuestos metálicos cuando se combinan con el metal químicamente corroído (Arvidson y

Johansson, 1977). Las propiedades electroquímicas de la saliva dependen de las concentraciones de sus componentes, pH, tensión superficial y su capacidad buffer (tampón). Con ello, podemos decir que la magnitud del proceso de corrosión resultante puede estar controlado por estas variables (Chaturvedi, 2008). Los productos que se liberan pueden ser tragados por el paciente, o bien, se pueden adherir a las superficies mucosas o dentarias (Janson y col., 1998),

Una serie de factores puede influir en la corrosión de una aleación que se utilice en materiales dentarios (Lucas y Lemons, 1992; Macedo y Cardoso 2010):

- Composición de la aleación (particularmente en la superficie)
- Fases en la estructura de la aleación-estructura de la superficie (rugosidad, presencia de óxidos)
- Superficie (fisuras, hoyos, etc.)
- Tratamiento térmico
- Combinaciones de aleaciones
- Tiempo de servicio

2.1. Tipos de Corrosión

Existen diversas formas de corrosión que afectan a las aleaciones utilizadas en arcos con fines ortodóncicos (Eliades y Athanasios, 2002):

1. Corrosión uniforme: ("Uniform attack")

Es el tipo de corrosión más común, que ocurre en todos los metales en diferentes cantidades. El proceso se origina a partir de la interacción de metales con el medio ambiente, produciendo la consecuente formación de hidróxidos y compuestos organometálicos. Un requisito fundamental en este tipo de corrosión es que el medio corrosivo debe tener el mismo acceso a todas las partes de la superficie, presentando el metal una uniformidad en cuanto a su composición y metalurgia. Este tipo de corrosión no se detecta hasta que se disuelven elevados niveles de metal (Chaturvedi, 2008; Eliades y Athanasios, 2002).

2. Corrosión por picadura/hoyo/fosa ("Pitting Corrosion")

Este es uno de los tipos de corrosión que tienen lugar en los brackets y arcos. Un hoyo/picadura/fosa se considera un poro con una profundidad igual a su anchura. Sorprendentemente, el proceso de corrosión comienza antes de ser

colocado en el medio intraoral ya que se han encontrado superficies porosas en productos sin utilizar (Papadopoulos y col., 2000). Las superficies de arcos de acero inoxidable y NiTi previos a ser utilizados presentan grietas y poros. Dichos poros favorecen el ataque ya que representan superficies susceptibles a la corrosión.

En arcos compuestos de acero inoxidable, CoCr, NiCr, NiTi, y ßTi expuestos a corrosión electroquímica en saliva artificial se han observado evidencias de la formación de "pitting corrosion" sobre sus superficies (Oshida y col., 1992; Barret y col., 1993). Estudios electroquímicos han demostrado, igualmente, que este tipo de corrosión ocurre en los arcos de NiTi en una solución salina al 1%. Sin embargo, recordemos que muchos de los poros presentes en este tipo de arcos proceden del proceso de fabricación (Brantley, 2000).

3. Corrosión de grietas ("Crevice Corrosion or Gasket Corrosion"):

Este tipo de corrosión ocurre entre dos superficies o en zonas constreñidas donde el intercambio de oxígeno no es posible (Chaturvedi, 2008). Tiene lugar al poner en contacto estructuras no metálicas y un metal, como es el caso de una ligadura elastomérica sobre un bracket, produciéndose debido a las diferencias en iones metálicos o en concentración de oxígeno entre la grieta y sus alrededores.

En el material clínico, la profundidad de las fisuras puede alcanzar 2-5 mm, perforando la base del bracket, llegando, por tanto, a alcanzar altos niveles de metales disueltos. El ataque puede producirse debido a la falta de oxígeno en asociación a la formación de placa y bioproductos de la microflora, los cuales agotan el oxígeno alterando la regeneración de la capa pasiva de óxidos de Cr (Olefjord y Wegrelius, 1990; Eliades y Athanasios, 2002).

La reducción del pH y el incremento en la concentración de iones cloruros son dos factores esenciales en la iniciación y en la propagación del fenómeno de corrosión de grietas. A medida que la acidificación del medio aumenta en el tiempo, la capa pasiva de la aleación se disuelve, acelerándose, con ello, el proceso de corrosión local (Chaturvedi, 2008).

La corrosión de grietas y la disolución de Ni de las regiones cercanas han sido investigadas, a su vez, en arcos de distintos materiales (Nitinol, acero inoxidable, Elastinol) *in vitro* por Grimsdottir y col. (1992). Sin embargo, estos cambios de composición se han registrado en grietas tanto en arcos de NiTi nuevos como los usados, implicando, por ello, la posibilidad de considerar los defectos de fabricación. Se observaron varias diferencias importantes en la morfología de la superficie entre ambos tipos de arcos. La superficie de las regiones acopladas a la ranura del bracket se mostraba excesivamente desgastada y se observaron patrones característicos de delaminación. El deterioro aumentado de esta región específica puede atribuirse a las fuerzas compresivas que se dan en la activación del arco a través del ligado y al posible daño friccional que se produce en el interior de la ranura.

4. Corrosión Galvánica ("Galvanic Corrosion"):

Este tipo de corrosión es la disolución de metales provocada por diferencias macroscópicas en sus potenciales electroquímicos, generalmente ocasionado por la proximidad de dos metales diferentes (Chaturvedi, 2008). Cuando dos o más metales distintos entran en contacto, o bien, las mismas aleaciones pero sujetas a diferentes tratamientos mientras se exponen a fluidos orales, tiene lugar un proceso combinado de oxidación y disolución debido a la diferencia entre sus potenciales de corrosión (Eliades y Athanasios, 2002). El metal menos noble se oxida y se convierte en ánodo, dando lugar a la liberación de electrones de algunos átomos y produciendo iones solubles (Merritt y Brown, 1995). El metal más noble se convierte en cátodo y es más resistente a la corrosión con respecto al menos noble (Eliades y Athanasios, 2002). En una situación clínica, este fenómeno tiene lugar al poner en contacto brackets y arcos ortodóncicos (Chaturvedi, 2008).

El acero inoxidable se caracteriza por un comportamiento activo-pasivo dependiendo de las condiciones medioambientales en las que la capa de óxido de cromo será eliminada (forma activa) o regenerada (forma pasiva). Por ello, la

corrosión galvánica tiene lugar dependiendo del estado del acero inoxidable (Eliades y Athanasios, 2002).

Reed y Willman (1940) demostraron por primera vez, con detalle, la presencia de corrientes galvánicas en la cavidad oral, estableciendo magnitudes aproximadas de los iones liberados. Iijima y col. (2006) investigaron la corrosión galvánica en la combinación de dos tipos de brackets (Ti y stainless steel, SS) con diversos materiales de arcos (NiTi, CrCoTi, SS y βTi). Demostraron que la unión de la aleación de NiTi con SUS 304 o Ti exhibía una densidad de corriente galvánica relativamente alta incluso tras 72 horas. Se ha sugerido que la combinación de SUS 304-NiTi y Ti-NiTi acelera marcadamente la corrosión de aleaciones de NiTi, actuando este último como ánodo. En la combinación de aleaciones de NiTi con Ti, el Ti actúa como ánodo y se corroe en los primeros estadíos; sin embargo, la polaridad es inversa después de la primera hora, dando lugar a la corrosión de NiTi. Las diferencias en las proporciones de áreas entre ánodo-cátodo utilizados en este estudio tuvieron muy poco efecto en el comportamiento de corrosión galvánica. Estudios previos (Iijima y col., 2001) han demostrado que la película de óxido existente en los arcos comerciales de NiTi, formada por el proceso de fabricación con calor, da lugar a un incremento de la resistencia a la corrosión.

Sin embargo, otros estudios anteriores (Yuasa y col., 2004) que miden el potencial de corrosión libre en una solución de NaCl al 0,9% en arcos ortodóncicos, brackets y coil Springs, demostraron que los brackets fabricados a partir de dos piezas a través de soldadura poseen un potencial de corrosión mucho menor en comparación con aquellos brackets de una sola pieza, ya que el punto de contacto entre la ranura y la base del bracket es susceptible de corrosión localizada.

5. Corrosión Intergranular ("Intergranular Corrosion"):

Los brackets de acero inoxidable que se encuentran bajo un rango de temperatura, conocidos como temperaturas de sensibilización, dan lugar a una

alteración de su microestructura. Este fenómeno se debe a la precipitación de carburo crómico en los límites de los *granos* (Eliades y Athanasios, 2002). Como resultado, los alrededores de los granos y regiones adyacentes poseen a menudo menor resistencia a la corrosión, provocando un mayor grado de corrosión en estas zonas (Chaturvedi, 2008). A diferencia de la corrosión uniforme y de fisuras, este tipo de corrosión afecta principalmente a la solubilidad del carburo de cromo (Eliades y Athanasios, 2002).

6. Corrosión fretting y erosión-corrosión ("Fretting and Erosion-Corrosion"):

La combinación de un fluido corrosivo y una elevada velocidad de flujo da lugar a fenómenos de erosión-corrosión. El propio estancamiento o la disminución de la velocidad de fluidos causarán una tasa de corrosión baja o moderada, pero el movimiento rápido del fluido corrosivo físicamente erosiona y elimina la película protectora a la corrosión, exponiendo la aleación reactiva subyacente y acelerando la corrosión.

La corrosión fretting es un tipo de erosión-corrosión. Se refiere al proceso que ocurre en las áreas de contacto de materiales bajo carga, encontrando su análogo en la interfase entre arco y ranura del bracket. Es la responsable de la mayor parte de liberación de metales en los tejidos, existiendo una acción conjunta de ataque químico y mecánico (Chaturvedi, 2008).

Resulta interesante resaltar que la aparición de esta corrosión es muy diferente de la superficie típica del arco producida en métodos de envejecimiento *in vitro* tras la aplicación de soluciones de saliva artificial o electrolíticas.

Alternativamente, la mayoría de los metales están cubiertos por una fina capa de óxidos que se altera y produce desechos de óxidos, produciendo una oxidación acelerada (Eliades y Athanasios, 2002).

7. Corrosión Microbiológicamente Influenciada ("Microbiologically influenced corrosion"):

Matasa (1995) fue el primero en mostrar evidencias del ataque microbiano a los adhesivos en el campo ortodóncico. Sin embargo, anteriormente se había informado acerca del efecto de la actividad enzimática y la degradación de las resinas de composite. La aplicación ortodóncica se caracteriza por la formación de cráteres en la base del bracket (Eliades y Athanasios, 2002).

Diferentes estudios corroboran que las bacterias sulfato y nitrato-reductoras son agresivas e inflamatorias para los tejidos huéspedes, así como el hecho de que estas bacterias también afectan los procesos de corrosión de diversas aleaciones (Margelos y col., 1991). Durante el ataque microbiano a las aplicaciones metálicas ortodóncicas se generan diversos ácidos en el medio intraoral. En la superficie de los dientes se crea un biofilm con la ayuda de restos alimenticios y productos del metabolismo microbiano. Los microorganismos aeróbicos utilizan azúcares simples en el proceso de glicolisis, liberando CO_2. Generalmente, los facultativos quimioorganotropos utilizan los azúcares y producen ácidos orgánicos, alcoholes y CO_2. En condiciones anaeróbicas, los quimioorganotropos, como las Bacteria Reductoras de Sulfato (Sulphate Reducing Bacteria, SRB) utilizan lactato como fuente de carbón, reduciendo el sulfato a sulfuro. Finalmente, el sulfuro se combina con iones de hierro para formar sulfuro ferroso como producto de corrosión final. En presencia de Bacterias Oxidativas de Sulfato (Sulphate Oxiding Bacteria, SOB), el sulfuro se oxida a sulfato. En la cavidad oral, el hidrógeno se combina con sulfato para formar ácido sulfúrico, siendo éste mucho más corrosivo. El pH reducido, producido por la formación de ácido, influye en la descalcificación de los dientes y en la corrosión de los aparatos ortodóncicos (Chaturvedi, 2008). Debido a la deposición del biofilm, la superficie del metal bajo éste está expuesta a diferentes cantidades de oxígeno en comparación a otras áreas. Las zonas con menor disposición de oxígeno actúan como ánodo, la cual da lugar a la corrosión, liberando iones metálicos a la saliva. Estos iones metálicos se combinan con los productos finales de las bacterias en la

saliva actuando ésta como electrolito, dando lugar a productos de mayor corrosión, como $MnCl_2$, $FeCl_2$, entre otros (Maruthamuthu y col., 2005).

La incidencia y la severidad de la corrosión microbiana puede reducirse manteniendo dicho área lo más limpia posible, así como con la utilización de sprays antibióticos, con el fin de reducir las poblaciones de microorganismos. Chang y col., (2003) demostraron el incremento de la corrosión de los materiales dentales metálicos en presencia de *Streptococcus mutans* y sus subproductos de crecimiento. Maruthamuthu y col., (2005) estudiaron el comportamiento electroquímico de los microorganismos en los arcos ortodóncicos en saliva artificial con y sin saliva. De acuerdo con su estudio, las bacterias reducen levemente la resistencia e incrementan la corriente de corrosión.

La liberación de Mn, Cr, Ni y Fe de los arcos ortodóncicos se debe a la existencia de factores oxidativos de Mn, factores oxidativos de Fe y bacterias heterotrópicas en la saliva (Chaturvedi, 2008).

8. Corrosión por estrés ("Stress Corrosion"):

Cuando ligamos un arco a brackets en casos de apiñamientos severos, se incrementa el estado de reactividad de las aleaciones. La reactividad aumentada resulta de la generación de estrés de tensión y compresión que se desarrolla localmente debido a la sobrecarga multiaxial y tridimensional del arco. En consecuencia, se produce una diferencia de potencial electroquímico, con unas zonas específicas actuando como ánodos y, otras, como cátodos (Eliades y Athanasios, 2002). Wang y col. (2007) estudiaron el agrietamiento debido a la corrosión por estrés del NiTi en saliva artificial y demostraron que los arcos ortodóncicos de NiTi se fracturaban por el agrietamiento de la corrosión por estrés durante su uso.

9. Fatiga de Corrosión ("Corrosion Fatigue"):

Un proceso importante en el envejecimiento de las aleaciones ortodóncicas es la tendencia del metal a la fractura bajo situaciones de estrés cíclico de

repetición. Este proceso de fatiga se acelera debido a la reducción de la resistencia inducida por la exposición a un medio corrosivo como la saliva. Este fenómeno ocurre frecuentemente en arcos que permanecen en el medio intraoral durante extensos periodos de tiempo bajo carga y, por lo general, se caracteriza por la suavidad en las áreas de fractura, las cuales incluyen, a su vez, zonas de rugosidad aumentada, así como de apariencia cristalina (Eliades y Athanasios, 2002). En la Figura 1 se muestra un esquema de los distintos tipos de corrosión anteriormente citados.

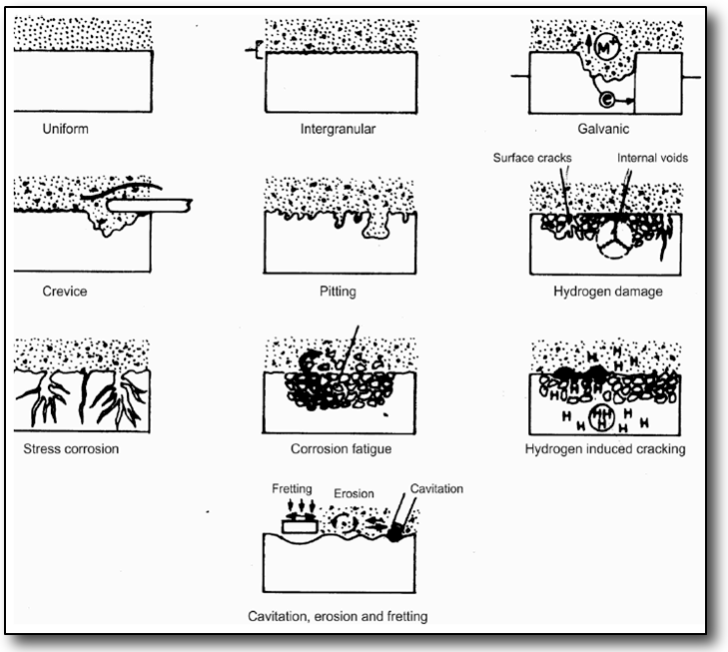

Figura 1. Diferentes tipos de corrosión que puede sufrir la aparatología ortodóncica (Chaturvedi, 2008).

2.2. Resistencia a la corrosión

Los estudios más recientes se han centrado en las alteraciones de arcos de NiTi, así como en el análisis de su superficie interna. Se ha encontrado que las superficies de los materiales están cubiertas de unos tegumentos proteínicos que enmascaran la topografía de superficie de la aleación, dependiendo de las condiciones medioambientales orales de cada paciente individualmente y del periodo de exposición intraoral. Estos filamentos proteínicos constituyen el

denominado biofilm. Los constituyentes orgánicos de la película adquiridos sobre la superficie de la aleación fueron amida, alcohol y carbonato, mientras que las especies elementales predominantes fueron sodio, potasio, cloruro, calcio y fósforo. La distribución elemental del biofilm consiste en la formación de cloruro sódico, cloruro potásico y el fosfato cálcico cristalino que, posteriormente, precipita en la superficie del arco. Las regiones mineralizadas proporcionan un efecto protector sobre el sustrato de la aleación, especialmente bajo condiciones de pH disminuido, en las que la corrosión de acero inoxidable y NiTi está aumentada (Eliades y Athanasios, 2002).

La adsorción y calcificación del biofilm proporciona una película interna protectora, reduciendo de este modo la incidencia de la respuesta inmune del huésped ya que se disminuye la exposición de la superficie de la aleación al ambiente oral. Esta formación del biofilm proporciona la denominada "Resistencia a la Corrosión".

El deterioro de la resistencia a la corrosión de los arcos ortodóncicos tiene dos consecuencias:

- Pérdida de las propiedades físicas, las cuales juegan un papel muy importante en el éxito del tratamiento clínico.
- Liberación de iones de Ni, los cuales pueden ser, como ha sido demostrado, tóxicos, e incluso, causar reacciones alérgicas.

Aunque las disoluciones para los ensayos suelen tener pH 6-7, en la cavidad oral el pH usualmente oscila entre 4-5,5, y después de una comida, en determinadas zonas incluso se logran pH inferiores. Por ello, se ha estudiado como varía la resistencia a la corrosión en función de la acidez del medio. En concreto, el estudio realizado por Huang (2003) demostró que la resistencia a la corrosión disminuye de forma significativa con la acidificación del medio en el que se encuentren. Se analizaron cuatro arcos de NiTi inmersos en saliva artificial Fusayama modificada, diferentes pH: 2.5, 3.75, 5.0 y 6.25, manteniéndolos a 37º durante 1, 3, 7, 14 y 28 días, y determinaron mediante Espectroscopía de absorción atómica (AAS) la cantidad de iones de Ni y Ti liberados. Se conluyó

que tanto la fabricación, el pH, como el tiempo de inmersión tenían una influencia estadísticamente significativa en la liberación de iones de Ni y Ti de los arcos NiTi sin utilizar. La liberación de los iones metálicos se incrementó con el tiempo de inmersión en todas las soluciones estudiadas, siendo la media de iones liberados mucho menor que en la solución a pH 2.5. En pH=2.5 la cantidad de iones de Ni liberados era de magnitud muy similar a la de los iones de Ti liberados; sin embargo, en la solución de pH ≥ 3, 75 la cantidad de iones de Ti liberados fue mucho menor en comparación con los de Ni, siendo en ambos casos inferiores a la concentración crítica considerada necesaria para producir alergia (600-2500 μg) y a la ingesta dietética diaria de 300-500 μg.

Las diferencias en la resistencia a la corrosión entre arcos de diferentes fabricantes pueden deberse a variaciones en la caracterización de su superficie, tales como la topografía de su superficie y técnicas de procesado para su producción. Los defectos preexistentes de fabricación en la superficie de los arcos de NiTi son sitios preferentes para la corrosión, mientras que los arcos de NiTi con una superficie más áspera no mostraron una liberación iónica mayor. Por último, en relación a la liberación de iones de Ti, la película pasiva (generalmente de TiO_2) de los arcos de NiTi resultó muy protectora frente a la corrosión en la saliva artificial levemente acidificada. En los arcos NiTi, los riesgos potenciales de la corrosión están ligados a los efectos del Ni, pero un aumento de la liberación de iones Ti indica el deterioro de la película protectora de su superficie, y ello puede conducir a un aumento de la liberación de iones Ni (Huang, 2003).

Las aleaciones de alto contenido en Au son extremadamente resistentes a la corrosión debido a su estabilidad termodinámica (Canay y Oktermer, 1992).

La resistencia a la corrosión en aparatología ortodóncica es importante para la prevención de la liberación iónica en la cavidad oral. Los brackets metálicos se diseñan con diferentes aleaciones de acero inoxidable entre la base y las aletas, siendo posteriormente unidos a través de aleaciones de plata, oro o Ni (Zinelis y col., 2004). La aleación de la base del bracket es un metal más suave

con el fin de que el descementado sea más fácil, mientras que el metal de las aletas del bracket tiene mayor dureza para soportar las fuerzas aplicadas con los arcos (Eliades y col., 2003a). Las diferencias de composición entre las estructuras, junto con la aleación de soldado, crea diferencias en sus potenciales de corrosión (Fontana, 1986). Con la finalidad de prevenir esta liberación iónica, se han introducido brackets de una sola unidad creados por inyección de metales (MIM brackets: "*Metal Injection Molding Brackets"*), que proporcionan una distribución uniforme de los elementos, eliminando, así, la posibilidad de corrosión galvánica que ocurre entre los componentes de los brackets (Siargos y col., 2007). Como el potencial de corrosión de los brackets depende de su composición, proceso de fabricación y microestructura, los brackets MIM se comportarán de forma diferente a los brackets convencionales. La aparatología fabricada por inyección metálica supone una mejora sobre los brackets convencionales ya que elimina el potencial de corrosión galvánica que ocurre entre el acero inoxidable y las aleaciones de soldado (Zinelis y col., 2005). Como resultado del proceso de fabricación y la microestructura formada, se reduce la liberación iónica así como las consecuencias biológicas adversas. En relación a la estructura interna de ambos tipos de brackets, los brackets convencionales muestran una porosidad menor y una consistencia más sólida, mientras que los brackets fabricados por inyección metálica poseen una cantidad de poros interna incrementada (Siargos y col., 2007). Por ello, aunque los brackets creados por inyección metálica están formados por una única unidad y están libres de corrosión galvánica, su aumentada porosidad incrementa su tendencia a la corrosión de fisuras ("*Pitting corrosion*") (Eliades y Athanasios, 2002; Zinelis y col., 2005).

Schiff y col. (2006) también estudiaron la resistencia a la corrosión de tres tipos de brackets (CoCr, FeCrNi y Ti) en combinación con tres enjuagues bucales. Los resultados demostraron que los materiales de los brackets se pueden dividir en dos grupos en función de su resistencia a la corrosión: Ti y FeCrNi en un grupo, y por otra parte CoCr, los cuales tienen propiedades parecidas al Pt. Muchos

estudios han demostrado que los iones fluoruros destruyen la capa protectora de TiO_2 en las superficies de las aleaciones de Ti, dando lugar a una morfología más susceptible de corrosión, menor resistencia de polarización y una mayor densidad de corriente anódica o liberación iónica (Huang y col., 2003).

3. LIBERACIÓN DE METALES A PARTIR DE APLICACIONES ORTODÓNTICAS

Diversos estudios han investigado si las aplicaciones de ortodoncia liberan iones metálicos a través de la emisión de corrientes electro-galvánicas, con la saliva como medio o a través de una continua erosión a lo largo del tiempo (Vandekereckhove y col., 1998). La liberación de un elemento es extremadamente difícil de predecir basándose en la composición de la aleación. Sabemos que la liberación del elemento no va en proporción a la cantidad del mismo en la aleación. Una aleación con un alto contenido en oro no libera necesariamente altas cantidades de dicho metal y una aleación con 1-2% de Zinc podrá liberar cantidades significativas del metal (Sáez y col., 1999). La biocompatibilidad de estos materiales está fuertemente relacionada con la liberación de iones y de ahí la preocupación del paciente por conocer la posible liberación de iones metálicos a partir de esta aparatología. La fatiga de las aleaciones produce unas tasas de liberación aceleradas, así como aumentos de las reacciones de desintegración (Fontana, 1986).

Las aleaciones ortodóncicas están en contacto con una variedad de sustancias que imponen potentes efectos en su estado reactivo y en la integridad de la superficie, entre ellas se encuentran:

·*Saliva:* Contiene derivados ácidos procedentes de la degradación y descomposición de comida.

·*Factores medioambientales*, como el aire.

·*Flora oral y sus subproductos*. Se ha demostrado la colonización y la simultánea precipitación de formaciones cristalinas, en su mayoría compuestas de complejos cálcico-fosforados. Se depositan especies estreptocócicas en la ligadura elastomérica tras la exposición intraoral.

La colonización microbiótica tiene dos funciones:

1. Algunas especies pueden metabolizar metales de las aleaciones.
2. Los subproductos microbianos y los procesos metabólicos pueden alterar las condiciones medioambientales.

Al implantar un material, ya sea puro o en aleación, en un medio *in vivo* fisiológico complicado y corrosivo, la estabilidad de la película de óxido de la superficie se afecta, quedando expuesta la superficie fresca de metal provocando liberación de gran cantidad de iones metálicos, aumentando dicha liberación (Matusiewicz, 2014).

Otro factor es la estructura de fases del material, de forma que en general, la presencia de múltiples fases incrementa el riesgo de liberación (Arenholt-Bindslev y col., 2009), y los elementos liberados parecen interrelacionarse en una aleación para influir en su liberación. La rugosidad de la superficie también incrementa la liberación de elementos porque las superficies rugosas tienen mayor área de exposición de los átomos al ambiente externo y crean microambientes locales que varían la exposición de la superficie a elementos como el oxígeno.

La aparatología fija (brackets, bandas, arcos y muelles) generalmente se compone a base de acero inoxidable, Ni-Ti o aleaciones Ni-Co. Los principales elementos que pueden liberarse son Fe, Cr, y Ni de los aceros y Ni y Ti a partir de las aleaciones Ni-Ti. Ni y Cr son los que han recibido la mayor atención en los últimos años por sus conocidos efectos adversos (Amini y col., 2008). Junto al Ni, los iones Co y Cr pueden causar hipersensibilidad y dermatitis, y estos elementos pueden inducir citotoxicidad y genotoxicidad. Los aceros inoxidables y aleaciones conteniendo Cr realmente no se corroen fácilmente, pues se crea una película de

óxido pasiva que ofrece protección frente a los iones agresivos, retrasando la corrosión. Sin embargo, cuando el acero se trata con calor, puede ocurrir oxidación en su superficie, de forma que cada tratamiento de calor y método de enfriamiento puede afectar al espesor de la película de óxido y dar lugar a varios grados de corrosión.

Los factores que influyen en los procesos de corrosión de diferentes aleaciones y consecuentemente en la cantidad de Ni liberada son: temperatura intraoral, pH, composición salivar, duración de la exposición, desgaste del arco debido a la fricción por mecanismos de deslizamiento, presencia de soldadura, tipo de arco, entre otros (Noble y col., 2008).

Los productos generados de la liberación de metales son absorbidos por el esmalte, como se demuestra en la incidencia de tinción dentaria que ocurre mediante la difusión a través de la capa adhesiva. "*Metallosis"* es la difusión de partículas metálicas generadas por reacciones que ocurren en el bracket (Maijer y Smith, 1982). Este fenómeno se transmite a la capa adhesiva evidenciándose en la Figura 2 (Eliades y Athanasios, 2002).

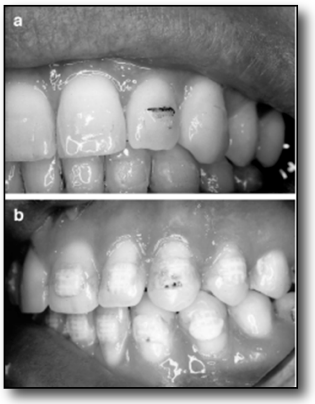

Figura 2. Apariencia del esmalte tras el descementado de un bracket metálico (a) y un bracket de plástico (b). La descoloración de la capa de adhesivo se atribuye a la difusión de productos de corrosión a partir de la base del bracket metálico o del arco de acero inoxidable, respectivamente (Eliades y Athanasios, 2002).

Las tinciones (negras o verdes), como observamos en estas situaciones, no son un fenómeno común en el campo de la ortodoncia. Se pueden dar numerosas posibilidades en relación a los productos de corrosión coloreados: los sulfuros y óxido de Ni son negros, mientras que el hidróxido de Ni es verde. El sulfuro de Cr es negro, y el fosfato de Cr es violeta. Tanto el óxido como el hidróxido de Cr son verdes. Por último el fluouro y fosfato de Ni son ambos verdes.

En el estudio realizado por Maijer y Smith (1982) las tinciones se observaron en la mayoría de los casos en los dientes anteriores. En todos los casos, las bases de los brackets estaban fabricadas con acero inoxidable de tipo 304. Aquellas bases construidas a partir de acero inoxidable tipo 306L no manifestaron tinción alguna. La escasa higiene oral que presentaron algunos pacientes podría haber influenciado indirectamente en el desglose de la capa de oxígeno protectora de la base del bracket. Por otra parte, podemos destacar que el esmalte de algunos dientes puede ser más susceptible a la tinción. El mecanismo que se produce en estos casos es la corrosión crevicular del acero inoxidable causada por la presencia de un diferencial de concentraciones de células que surgen de áreas con cementado defectuoso entre la base del bracket y la superficie del diente. Maijer y Smith determinaron que la causa primaria era el tipo de aleación, aunque otros factores, tales como la acción galvánica, el diseño de la base del bracket y su fabricación, así como el medio oral particular también contribuyen. El reciclado térmico de los brackets juega un papel importante como factor etiológico, ya que produce alteraciones de la microestructura metalúrgica. En el reciclado se aplican temperaturas excesivamente altas (600º y 800º) produciendo una precipitación de carburo de cromo en las zonas adyacentes a los granos de la aleación, desestabilizándola y disminuyendo la resistencia a la corrosión.

En relación al reciclado de los brackets debemos destacar el estudio realizado por Huang y col. (2004), que compararon la liberación de iones metálicos de brackets nuevos y reciclados, inmersos a distintos pH (4.0 y 7.0)

durante un periodo de 48 semanas. Los brackets metálicos inmersos en una solución tampón durante 48 semanas liberaban mayor número de iones que aquéllos inmersos menor tiempo. Este resultado era independiente del pH de la solución y de si el bracket era nuevo o de reciclado, demostrando que la corrosión de la superficie del metal se incrementaba en el tiempo. Dichos autores encontraron que los brackets metálicos inmersos en una solución a pH 4.0 liberaban más iones que aquéllos inmersos en saliva artificial a pH 7.0 y que dicha liberación se incrementaba con el tiempo de inmersión, siendo el Ni el ión liberado de forma predominante. Por último, determinaron que los brackets que se utilizan en un tratamiento convencional sufren corrosión tanto en medio ácido como neutro, siempre y cuando se encuentren inmersos durante un periodo prolongado de tiempo.

Se ha demostrado que el empleo de pasta de dientes (Wataha y col, 2003) y enjuagues bucales (Mueller, 1982) aumentan la liberación de iones de Ni de aleaciones Ni. Diversos estudios *in vitro* sobre el efecto del cepillado dental, han demostrado aumentos significativos en la liberación de elementos a partir de las aleaciones de Ni cuando se utilizaban pastas dentífricas; sin embargo, cuando el cepillado se realizaba en ausencia de pasta no se producía ningún incremento (Schiff y col., 2004). Se ha demostrado que los arcos ortodóncicos de NiTi en combinación con un medio de fluoruro liberaron una cantidad significativamente mayor Ni a la saliva artificial (Cioffi y col., 2005). Sin embargo, la exposición a fluoruros en la cavidad oral se produce durante cortos periodos de tiempo, no días. Por ello, para mejorar la simulación de las condiciones de la cavidad oral, es conveniente realizar exposiciones de corta duración de los arcos de NiTi en un medio fluorado. Los arcos de NiTi, especialmente aquéllos que contienen Cu, sufren una mayor corrosión en presencia de enjuagues fluorados (Schiff y col., 2006). Los resultados permiten ayudar a decidir en la práctica el tipo de enjuague

bucal que se debe prescribir a los pacientes, dependiendo de la fase del tratamiento en que se halle y de la aleación.

Los medios que contienen fluoruros penetran en los espacios estrechos existentes entre arco y bracket, donde no se puede llevar a cabo una completa limpieza. Las concentraciones tópicas de alto contenido en fluoruro se disponen en dichos espacios y atacan la interfase arco/bracket, en función de la concentración de fluoruros. Este fenómeno incrementa la fricción entre ambas superficies. La utilización de agentes tópicos fluorados al mismo tiempo que arcos NiTi puede disminuir las propiedades mecánicas y funcionales de los arcos y contribuir a un mayor tiempo de tratamiento ortodóncico (Walker y col., 2005).

3.1. Tipos de Estudios: *In-Vivo e In-Vitro*

Numerosos ensayos *in vitro* e *in vivo* han investigado la liberación metales, especialmente Ni y Cr.

3.1.1. Estudios *IN VITRO*

Los ensayos *in vitro* realizan mediciones de niveles de metales en medios simulados que consisten en soluciones electrolíticas y ácidas (solución de cloruro de sodio), saliva y sangre. El medio simulado denominado "Saliva Artificial" tipo Fusayama modificada es uno de los medios más usualmente empleados en este tipo de estudios. Existen una serie de factores y variables responsables de dichas diferencias:

·La ausencia de variaciones extremas en cuanto a los parámetros que afectan al potencial de corrosión, así como la reactividad de la aleación (pH, temperatura, estrés). El potencial de corrosión del acero inoxidable se aumenta en medios ácidos.

·La ausencia del ligado entre bracket y arco. Estos dos componentes son elementos móviles que inducen la "fretting corrosion" (Fontana, 1986).

·La ausencia de la flora intraoral, acumulación de placa y los subproductos. Esta es la diferencia más importante.

·El uso de disoluciones de almacenamiento estáticas, puede dar lugar a una falsa evidencia. La liberación de Ni de los arcos en estas condiciones da lugar a una rápida obtención de equilibrio en la disolución, no teniendo en cuenta la liberación a largo plazo (Eliades y Athanasios, 2002).

Los estudios *in vitro* presentan varios inconvenientes, debido a sus simplificaciones y adolecen de falta de relevancia clínica. La estimación de la liberación iónica a través del uso de un medio de almacenaje compuesto por una solución que no se agita (estática), que no se repone, no proporciona solidez metodológica a la prueba, según Eliades y Athanasios (2002) En estas condiciones, la tasa de liberación está forzada a alcanzar más rápidamente una meseta ya que se establece un equilibrio entre los iones metálicos presentes en la disolución y los iones metálicos en la interfase metal-disolución. Este fenómeno nos dirige a la falsa conclusión de que la tasa de liberación se acelera al inicio y permanece constante posteriormente. Esta observación se contrapone a los resultados de muchos estudios que demuestran que el envejecimiento de las aleaciones en forma de fatiga o corrosión mejora la liberación iónica (Fontana, 1986).

Por contraste con los ensayos *in vivo*, los ensayos *in vitro* indican un potencial tóxico claramente dosis-dependiente por exposición al Ni. La exposición en condiciones ácidas, y dinámicas, en comparación con las condiciones estáticas (Kerosuo y col., 1995) pueden incrementar la liberación de iones Ni a partir de aleaciones de Ni.

Revisando la literatura científica, exponemos los resultados más significativos en relación a este tipo de estudios *in vitro*.

Grimsdottir y col. (1992) estudiaron la liberación de iones Ni y Cr a partir de aparatología ortodóncica: anclaje extraoral, bandas, brackets y arcos. Obtuvieron como resultado que la mayor cantidad de Ni liberado a los 14 días

procedía de los anclajes extraorales, los cuales contenían una elevada cantidad de soldadura de plata. Esto concuerda con lo citado por Berge y col.(1982), quienes introdujeron que la soldadura de plata crea una pareja galvánica, influenciando tanto en la soldadura como en el acero inoxidable. La pareja galvánica facilita la liberación de Ni, así como de otros metales. Hay que indicar que la sumersión en una solución de NaCl al 0,9%, posee una salinidad bastante elevada en relación a la saliva. Por otra parte, este estudio hizo uso de condiciones estáticas, sin embargo, cuando dichos elementos son utilizados en la cavidad oral se produce una activación mecánica para conseguir el movimiento dentario, es decir, es una situación dinámica. Por lo tanto, los movimientos relativos de arcos y la fricción que se producen dan lugar a otros tipos de corrosión, como es la "fretting corrosion", la cual favorece la liberación de los constituyentes de los aparatos (Grimsdottir y col., 1992). Como se ha comentado anteriormente, se concluye que la liberación de Ni y Cr está relacionada con la composición y el proceso de fabricación, pero no directamente con el contenido en estos elementos.

Park y Shearer (1983) demostraron una liberación de 40 µg de Ni y 36 µg de Cr al día a partir de una aparatología fija completa ortodóncica. En su simulación, emplearon una solución de 0,05% de NaCl. Los signos iniciales de corrosión fueron notables a partir del día 3. Un análisis macroscópico de los aparatos reveló que la corrosión ocurría en las zonas de soldadura de la banda y, una vez iniciada, aumentaba de forma severa progresivamente. Por otra parte, no observaron evidencia de corrosión en los brackets cementados. Observaron que el volumen de Ni liberado permanece en la solución, mientras que una gran cantidad del Cr liberado estaba presente en un precipitado que se formaba durante la corrosión. La cantidad de Cr analizado en el precipitado era 18 veces mayor que el Ni precipitado. Con ello, deducen que el Ni liberado es un compuesto soluble mientras que el Cr se libera en una forma insoluble.

En condiciones de laboratorio, se ha demostrado que los tratamientos de calor de las aleaciones incrementan marcadamente la liberación de los iones metálicos por factores que oscilan entre 15-60 veces (Gjerdet y Hero, 1987).

Barrett y col. (1993) evaluaron los niveles de corrosión de la aparatología ortodóncica estándar concluyendo que libera cantidades detectables de Ni y Cr cuando son tratadas en medio de saliva artificial; que la liberación de Ni alcanza su máximo a la semana de la colocación de la aparatología, disminuyendo la tasa de liberación posteriormente, con el tiempo. Sin embargo, la liberación de Cr aumenta durante las dos primeras semanas y va disminuyendo los niveles de liberación a lo largo de las dos siguientes semanas. Estos autores establecieron que la liberación de Ni es 37 veces mayor que la del Cr.

Iijima y col. (2006) realizaron un estudio con el fin de cuantificar el comportamiento de corrosión entre diversas parejas de brackets y arcos de distintas aleaciones. Utilizaron dos tipos de materiales de brackets: SS y Ti; mientras que de arcos ortodóncicos utilizaron NiTi, SS, CoCrNi y ®Ti, combinándolos en distintas proporciones de superficies y sumergiéndolos por inmersión en una solución de NaCl al 0,9% durante 3 días sucesivos. En todas las combinaciones de parejas la densidad de corriente galvánica disminuía en el tiempo y permanecía casi constante tras 24 horas. En la combinación de NiTi con Ti o con ®Ti, es el Ti el que se comporta como ánodo en los estadíos iniciales y se corroe. Sin embargo, en el tiempo de una hora la polaridad se invierte, resultando en la corrosión del NiTi. Este fenómeno puede ser explicado por el momento en el que el potencial de corrosión varía, ya que éste en el caso del Ti era menor que el de NiTi inmediatamente tras la inmersión, siendo inversa tras 1 hora. Sugirieron que las combinaciones de SS 304-NiTi y NiTi-Ti aceleran la corrosión de la aleación de NiTi, actuando éste como ánodo, ya que el NiTi combinado con dichas aleaciones exhibe una densidad bastante mayor incluso tras 72 horas.

Gil y col. (1998) propusieron una técnica con el fin de mejorar los niveles de fricción de los arcos de NiTi, la denominaron "Nitruración Gaseosa". En su investigación, estudian la liberación de iones al medio salivar, tanto de la aleación nitrurada como del material en estado de recepción, utilizando aleaciones de NiTi cuya composición química fue 44% Ti y 56% Ni. Los ensayos se realizaron en saliva artificial a 37º, extrayéndose 10 mL de solución an diferentes tiempos con el fin de analizar la liberación de iones metálicos. Comprobaron que la nitruración gaseosa es un eficaz método de mejora del deslizamiento de los alambres ortodóncicos de NiTi superelástico respecto al bracket. Mediante microanálisis de energía dispersiva de Rayos X, se comprobó que la capa estaba compuesta principalmente por nitruros de titanio que son los que le confieren una elevada dureza y oxinitruros de titanio. Se apreció que los iones Ti y Ni liberados al medio salivar eran menores para el caso del material que presenta la capa nitrurada respecto al que no presentaba dicha capa. La curva que presenta la liberación de iones era, para ambos elementos, de tipo potencial que llegaba hasta valores casi de saturación o de crecimiento ralentizado cuando los tiempos de exposición fueron superiores (Saez y col., 1999). La capa de nitruros de titanio ofrecía un importante obstáculo a la liberación de los iones de Ti, ya que éstos están enlazados de manera covalente con el nitrógeno; este tipo de enlace presenta una mayor energía de enlace que el enlace metálico y, por tanto, habrá una mayor estabilidad. Además, los iones Ni tienen una mayor dificultad de salir de la aleación ya que en la superficie hay una capa de nitruros que dificulta su cinética de liberación. Esta capa de nitruros hace que el medio fisiológico no esté en contacto con los iones Ni ya que la capa superficial es de nitruros de Ti y por tanto la aparición de iones Ni se produce cuando acaba la capa de nitruros de Ti. Es de esperar, que la capa de nitruros de Ti no solamente mejore la liberación iónica sino también la resistencia a la corrosión del metal ya que esta capa es inerte al ataque de los electrolitos que tenemos en boca y actúa como material cerámico (Sáez y col., 1999).

Gil y col. (2004) realizaron un nuevo estudio acerca de la influencia del tratamiento de nitruración gaseosa en arcos NiTi y NiTiCu. En este estudio obtuvieron un comportamiento muy similar entre ambos materiales; en ambos casos, la concentración de iones liberados al medio se incrementó muy rápidamente al principio y, posteriormente, alcanzó un nivel de saturación.

Eliades y col. (2004) caracterizaron *in vitro*, de forma cualitativa y cuantitativa, los iones liberados a partir de series de brackets (20) de acero inoxidable y de arcos de Ni-Ti (dos grupos de10 arcos), qu sumergieron en disolución salina al 0,9% durante 1 mes. Se analizó el medio de inmersión mediante Espectrometría de emisión atómica con plasma acoplado por inducción (ICP-AES). Los resultados indicaron que no existía liberación de iones a partir de arcos Ni-Ti y en el grupo de brackets de acero inoxidable se detectaron trazas de Ni y de Cr. Por tanto, aunque los arcos de NI-Ti tienen un mayor contenido de Ni (45-50% en comparación con el 8-14% del acero inoxidable) y sustancialmente una mayor relación superficie/volumen que los brackets de acero, no se evidenció liberación de Ni; este hecho se atribuye a la capa de precipitado de oxido de Ti sobre la superficie de la aleación, que actúa como barrera para la difusión del Ni sobre la superficie, minimizando su reactividad con el ambiente que le rodea.

Huang y col. (2004) demostraron que el bracket metálico Ormco fue el que mayor concentración de Ni liberaba en medios de inmersión, y el bracket Tomy liberó menores cantidades de Ni, Cr y Cu en comparación con el resto de brackets metálicos.

Luft y col. (2009) investigaron en un ensayo de inmersión estático, la liberación de iones Ni (ICP-MS), a partir de 9 sistemas de brackets de diferentes materiales y diseños, y realizaron un estudio de las alteraciones de la superficie de los brackets tras la corrosión mediante microscopía electrónica de barrido. La liberación de ión Ni varió entre 0,01 µg/día hasta un máximo de 5,24 µg/día. Todos los materiales mostraron trazas de corrosión tras el ensayo electroquímico. Sin embargo, tras el ensayo de inmersión estática, sólo se detectaron defectos

menores de corrosión uniforme, o pitting corrosion, estando la mayoría de los defectos localizados en las bases de los brackets y en el hook y las cantidades de Ni liberadas fueron bajas, inferiores a la ingesta diaria dietética de Ni (300-500 μg); en algunos casos los cambios de superficie se correlacionaron con la cantidad de Ni liberado.

De forma global, en la excelente revisión sistemática acerca de la liberación de iones metálicos a partir de aplicaciones ortodóncicas llevada a cabo por Mikuliewicz y Chojnacka (2011a) los autores concluyen que los ensayos *in vitro* más usuales son los llevados a cabo en saliva artificial, NaCl 0,9% o bien ácidos orgánicos (ej. ácido láctico), sumergiéndose los materiales y posterior cuantificación de los iones metálicos por diferentes técnicas (ver apartado 3.2.). En la Tabla 2 se exponen los estudios recogidos en dicha revisión, con indicación del material ensayado, medio experimental, metales investigados, y concentraciones halladas. Se puede observar que muchos de estos estudios emplean materiales y metodologías diferentes, con variados líquidos de inmersión, métodos analíticos de determinación diferentes, y además los materiales ensayados tienen distinta procedencia industrial. En consecuencia, todos estos factores hacen que sea difícil el poder comparar estos estudios entre sí. Por ello se recomienda elaborar unos procedimientos de trabajo estandarizados (medio inmersión-tipo y volumen, condiciones de incubación, condiciones estáticas/dinámicas, y duración del experimento) para obtener resultados potencialmente comparables.

Además, sería muy adecuado completar y actualizar la revisión de los estudios *in vitro* existentes hasta la fecha actual.

Tabla 2. Concentración de metales liberados *in vitro* en medios de inmersión (nanogramos/mL) (tomada de Mikuliewicz y Chojnacka, 2011, modificada y contrastada con artículos originales).

Referencias	**Material**	**Medio de inmersión**	**Concentración de iones metálicos ± SD**							
			Cu		**Cr**		**Fe**		**Ni**	
			Experimental	**Control**	**Experimental**	**Control**	**Experimental**	**Control**	**Experimental**	**Control**
Park and Shearer 1983	Brackets, bandas, arcos	NaCl 0,05%	-	-	28.000±4500	ND	-	-	31.250±5,500	ND
Barret y col. 1993	Brackets, bandas, arcos	Saliva artificial	-	-	Arcos SS*: 21,2-233,1 Arcos Nitinol: 16,4-126,9	-	-	-	Arcos SS: 1.260-7.520 Arcos Nitinol: 702-8,408	-
Kerosuo y col. (1995)[a]	Brackets, bandas, arcos	NaCl 0,9%	-	-	Estático: 4,5±2 Dinámico: 2,5±0.7	-	-	-	Estático: 17,1±3.4 Dinámico: 44,3±22.8	-
Staffolani y col., 1999[b]	Brackets, bandas, arcos	HCl y ácidos orgánicos (TCA y LA)[b]	HCl: 0.27- 10.88 Ácidos orgánicos: 1,69-3,79	ND	HCl:0,36 - 3.36 Ácidos orgánicos: 2,92-3.49	ND	-	-	HCl: 1.62- 8.73 Ácidos orgánicos: 8.40-10.49	ND
Hwang y col. 2001	Brackets, bandas, arcos. A y B: arcos SS C y D: arcos NiTi	Saliva artificial	(C) 7,5	ND	(A) 900 (B) 687±8 (C) 10,4 (D) 20,2	ND	(A) ca. 7800 (B) ca. 3100 (C) ca. 720 (D) ca. 775	ND	(A) ca. 800 (B) ca. 430 (C) 17,0±0.5 (D) ca. 35	ND
Eliades y col. 2004	Brackets Arcos	NaCl 0,9%	-	- -	1000	- -	-	- -	11000±900	- -
Gürsoy y col. 2004[c]	Brackets, bandas, arcos	Saliva artificial	(A) 10±3 (B) 15±5 (C) 25±6 (D) 31±10	-	(A) 4.5±0.5 (B) 4,0±0,7 (C) 7,3±0.6 (D) 7,5±0.6	-	(A) 67,7±9.6 (B) 64,3±8 (C) 115±12 (D) 140±9	-	(A) 10±6 (B) 15±3 (C) 20±6 (D) 20±3	-

Darabara y col. 2007[d]	Brackets, arcos	Ácido láctico 1M	-	-	(A) 1,18 (B) 6,48 (C) 1,02 (D) 1,68 (E) 0 (F) 0	-	-	-	(A) 43,9 (B) 44,7 (C) 53,4 (D) 45,5 (E) 44,8 (F) 16,9	
Kuhta y col. 2009	Brackets, bandas, arcos: stainless steel (SS); NiTi; y termo NiTi (T-NiTi)	Saliva artificial. pH 3,5 y pH 6,75. durante 28 días	(NiTi total$_{3.5}$) 15,123 (NiTi total$_{6.75}$) 546 (T-NiTi total $_{3.5}$) 18,588 (T-NiTi total$_{6.75}$) 516 (SS total$_{3.5}$) 19,893 (SS total$_{6.75}$) 708	-	(NiTi$_{3.5}$) 3,758 (NiTi$_{6.75}$) 57.8 (T-NiTi$_{3.5}$) 3,293 (T-NiTi$_{6.75}$) 31.2 (SS$_{3.5}$) 4,307 (SS$_{6.75}$) 76.2	-	(NiTi$_{3,.5}$) 15,489 (NiTi$_{6.75}$) 387 (T-NiTi$_{3.5}$) 13,432 (T-NiTi$_{6.75}$) 232 (SS$_{3.5}$) 15,150 (SS$_{6.75}$) 406	-	(NiTi$_{3.5}$) 5,430 (NiTi$_{6.75}$) 167 (T-NiTi$_{3.5}$) 4,553 (T-NiTi$_{6.75}$) 119 (SS$_{3.5}$) 5,611 (SS$_{6.75}$) 194	-

ND: No disponible; ca: valor tomado gráficamente

[*] SS: Stainless steel

[a] Expresado como microgramos/aparatología (inmerso en 15 ml medio, cantidades acumuladas en 8 días). TCA = disolución de ácidos tartarico, citrico y ascórbico, pH 2,2; LA= disolución de ácidos láctico y acético, pH:2,5

[b] Expresado como microgramos/aparatología (inmerso en 100 ml medio, cantidades acumuladas)

[c] A: controles; B: Brackets nuevos y arcos reciclados; C: brackets reciclados y arcos nuevos; D: brackets y arcos reciclados.

[d] A-F: Cobre NiTi arcos y seis diferentes brackets

3.1.2. Estudios IN VIVO

La humedad y las condiciones húmedas en el interior de la cavidad oral ofrecen un medio ideal para la biodegradación de metales, facilitando consecuentemente la liberación de iones que ocasionan efectos adversos. Ya se han indicado los inconvenientes de los ensayos *in vitro* de liberación de metales, en comparación con las condiciones de una situación clínica de rutina.

Los métodos *in vivo* más relevantes clínicamente suponen la estimación de liberación del elemento metálico en cuestión (Ni, Cr, Co, etc) en fluidos biológicos, como saliva, suero y orina, inicialmente, aunque estas matrices han ido evolucionando. La saliva es la primera fuente de disolución del elemento metálico, y los resultados tienen asociación directa con la cantidad de metal liberado. Las concentraciones de metal en suero y orina, sin embargo, dependen de la tasa de excreción del elemento en cuestión, por ej. Ni, siendo un parámetro muy individualizado así como específico de cada especie. Eliades y Athanasios (2002) indicaron que las ecuaciones para predecir el contenido total de metal en el organismo no tienen en cuenta la presencia de componentes multicompartimentales en el mismo, así como tampoco la unión selectiva de los metales a cada órgano.

Investigaciones *in vivo* han indicado una concentración en saliva de Ni y Fe mayor a las tres semanas tras la colocación de aparatología fija ortodóncica (Agaoglu y col., 2001). Sin embargo, otros autores indican que el hecho de que existan innumerables variaciones derivadas de la alta diversidad en el número de bandas y brackets de cada participante, hace que no existan diferencias estadísticamente significativas en las concentraciones de Ni (Gjerdet y col., 1991). En el mismo sentido, estudios de los contenidos de Ni y Cr en saliva no revelaron una concentración aumentada de dichos iones en un periodo que comprendía desde el primer día hasta un mes posterior a la colocación de aparatología fija en relación a las concentraciones previas a la inserción (Kerosuo y col., 1997). Según estos autores, los periodos que se adoptaron en la toma de muestras de saliva no excedían en un mes, intervalo 20 veces menor de un tratamiento

ortodóncico típico, siendo este un factor importante en la falta de tasas de liberación significativas. Como resultado, el efecto de los procesos de corrosión y el fenómeno mecánico como el desgaste o la fatiga en la liberación de Ni podría no ser explicado. La toma de muestras de saliva en diversos estudios fue llevada a cabo en puntos de tiempo discretos, dando lugar a una notable falta de datos acumulados y continuos que se extiendan a lo largo de un amplio periodo de tiempo (Gjerdet y col., 1991; Kerosuo y col., 1997).

Por otra parte, en ocasiones el protocolo de la toma de saliva, que involucra la estimulación a través de la masticación de una pieza de cera de parafina, inevitablemente restringe la colección de saliva a aquélla que se secreta casi directamente de la glándula salival. Este efecto surge por la falta de saliva que empapa la cavidad oral, incluyendo los dientes, limitando, por tanto, la exposición de la saliva que se segrega a la aparatología ortodóncica. Este patrón de liberación tan corto posee un valor predictivo muy pobre para el potencial de liberación a largo plazo.

Bishara y col. (1993) investigaron las concentraciones de Ni en sangre durante los estadíos iniciales de un tratamiento ortodóncico, concluyendo que los pacientes con aparatología fija ortodóncica no mostraron un aumento significativo de Ni en sangre en los primeros 4 y 5 meses de la terapia ortodóncica.

Diversas investigaciones han fallado en demostrar un incremento significativo de iones Ni y Cr en saliva de pacientes tratados un mes después de la inserción de la aplicación en comparación con los niveles antes de la inserción (Gjerdet y col., 1991; Kerosuo y col., 1997). Mientras otros estudios informan de un incremento de la concentración en saliva de Ni y Cr tras la inserción de las aplicaciones (Kocadereli y col., 2000; Eliades y col., 2003b; Fors y Persson, 2006).

En definitiva, son diversos los estudios *in vivo* realizados que han evaluado la cantidad de metales liberados a partir de aplicaciones en ortodoncia bajo diferentes condiciones físicas y químicas, existiendo una disparidad en los criterios de experimentación y de obtención de resultados (Gjerdet y col., 1991; Bishara y col., 1993; Kerosuo y col., 1997; Kocadereli y col., 2000; Agaoglu y col. 2001; Eliades y col.,

2003b; Fors y Persson, 2006; Amini y col., 2008; Petoumeno y col., 2008;Matos de Souza y col., 2008).

Los diferentes estudios *in vivo* sobre liberación de metales por aparatología ortodóncica han sido revisados de forma sistemática por Mikuliewicz y Chojnacka (2010), y Matusiewicz y col. (2014). Estos autores estudiaron la liberación a partir de implantes en general, con un apartado específico dedicado a las aplicaciones ortodóncicas En la Tabla 3 se recoge un resumen de dichos artículos, con la matriz y elementos investigados, y las concentraciones halladas. Se confirma que la mayoría de las investigaciones se centran en la liberación de Ni y Cr, seguido de Co, Fe, Ti y molibdeno (Mo), en matrices diversas como sangre, suero, orina y saliva, por periodos de tiempo comprendidos entre 1 día y 1-2 meses. De forma general se concluye que los iones metálicos se liberan al inicio del tratamiento. Y los autores indican la necesidad de llevar a cabo estudios *in vivo* a largo plazo, que monitoricen de forma crónica la potencial liberación de metales. Se indica además la inexistencia de investigaciones en matrices como pelo, que nos indicarían posible bioacumulación a largo plazo.

3.2. Técnicas analíticas, matrices y procedimientos para la evaluación de la liberación de metales a partir de aparatología ortodóncica.

3.2.1 Métodos de análisis.

La elección de un método apropiado de análisis depende de varios factores: el tipo y tamaño de muestra a analizar, los elementos a medir, la cantidad y rango de concentraciones, la exactitud y precisión, velocidad y coste del análisis, disponibilidad de instrumentación etc. Varios de los elementos liberados a partir de la aparatología ortodóncica van a estar presentes en los fluidos biológicos y tejidos a concentraciones o cantidades que pueden estar ligeramente por debajo o ligeramente por encima de los límites de detección (LODs) de la técnica en cuestión. Por ello, en estos casos, el cociente señal/ruido puede ser bajo, lo que lleva a que los valores medidos estén sujetos a variabilidad (Matusiewicz, 2014), de ahí la necesidad de disponer de métodos y/o

combinación de métodos que nos permitan el análisis multielemento con gran precisión y exactitud.

La liberación concreta de elementos metálicos a partir de aplicaciones ortodóncicas en diferentes matrices ha sido llevada a cabo de forma rápida y segura mediante Espectroscopía de Absorción atómica (AAS) (Amini y col., 2012), incluyendo su variedad electrotérmica, también denominada horno de grafito (GF-AAS) (Amini y col., 2008). Asimismo, se ha aplicado de forma satisfactoria la Espectroscopia de emisión atómica de plasma acoplado inductivamente (ICP-OES) (Eliades y col., 2004; Mikuliewicz y col., 2014), o bien Espectrometría de Masas con Plasma Acoplado Inductivamente (ICP-MS) (Liu y col., 2011; Reimann y col., 2012; Mikuliewicz y col., 2012). Para muchos elementos, el poder de detección de ICP-OES no es suficiente para determinar las concentraciones de elementos de fondo. En general, se obtienen mejores y mas bajos LODs por ICP-MS en comparación con ICP-OES (Heitland y col., 2006), por lo que su aplicabilidad ha sido limitada, y sobrepasada por otras técnicas más robustas, como GF-AAS y ICP-MS. De forma general, la aplicación de ICP-MS permite una más rápida y segura determinación de multielementos rutinaria en muestras biológicas de forma segura, debido a las mejoras de su sensibilidad y robustez.

En las Tablas 2 y 3 se puede comprobar y consultar la aplicabilidad de las técnicas de AAS, GF-AAS, ICP-OES e ICP-MS en la determinación de elementos metálicos liberados, especialmente Ni, Cr, Co, Fe.

3.2.2. Matrices y procedimientos de preparación

Los tipos de matrices a los que se pueden aplicar estas técnicas, junto a los parámetros más importantes seleccionados para la determinación de estos elementos en fluidos biológicos han sido revisados recientemente por Matusiewicz (2014) para implantes en general, resumiéndose las ventajas e inconvenientes de cada técnica. En las tablas anteriormente mencionadas, se puede comprobar que las matrices empleadas en los ensayos *in vivo* han sido: fundamentalmente saliva (Agaoglu y col., 2001; Eliades y col., 2003b; Amini y col., 2008), y en menor medida suero (Agaoglu y col., 2001), orina

(Menezes y col., 2007) y células de mucosa oral (Faccioni y col., 2003).

La saliva, a pesar de ser una muestra con indudables ventajas, como son: muestra no invasiva, de elección para niños, coste bajo, sin riesgo de infecciones y sin requerimientos especiales de manejo y conservación (Nriagu y col., 2006), también posee importantes inconvenientes. Su principal desventaja en relación con las células de la mucosa oral, por ejemplo, es que su flujo está influido por muchos factores, no afectándose por las concentraciones de las sustancias en un mismo grado, por lo que es útil para monitorizar productos químicos que no dependan de su flujo (Esteban y Castaño, 2009). Además, la información que proporciona es exclusiva del momento del muestreo (Hafez y col., 2011). Se considera totalmente necesario el desarrollar más métodos que determinen la liberación de metales en células de la mucosa oral, ya que las mismas están en contacto directo con el material ortodóncico, y su obtención es no invasiva (Mikulewicz, y col., 2011).

En este tipo de determinaciones las fuentes potenciales de contaminación han de identificarse y eliminarse, por lo que es imperativo mantener un control completo sobre la alta pureza de reactivos y de agua empleados en la preparación de las muestras. Evitar la adsorción de los metales traza en las pareces de los contenedores es otra cuestión a tener en cuenta. Es imprescindible controlar la contaminación desde la obtención de la muestra, ya que dicha contaminación puede originarse a partir del material del contenedor (Al en vidrio), del uso de anticoagulantes, y del instrumental empleado en la recogida de muestras, debiéndose utilizar jeringas o espátulas de plástico, en lugar de metálicas. Por ejemplo, si el material esta constituido por acero inoxidable, hay un peligro potencial de liberación de Fe, Cr y Ni. La recolección de las muestras ha de ser muy minuciosa, debiéndose recoger en envases de Teflon, polietileno, o polipropileno, que han debido ser tratados con medio ácido antes de su uso. Posteriormente, las muestras deben almacenarse bien a temperaturas de refrigeración o de congelación a -20ºC.

La elección del método de preparación de la muestra es primordial en Espectroscopía atómica (AAS, ICP-OES, ICP-MS), ya que intervienen importantes puntos: 1) la naturaleza de la muestra biológica en los ensayos *in vivo*; 2) la técnica

analítica empleada para la determinación del analito; 3) el número de muestras; 4) el analito y el rango de concentraciones esperado del mismo; 4) la certeza y precisión requeridas; 5) el tiempo de pretratamiento de la muestra (Matusiewicz, 2014). Por ej., la alta viscosidad de algunos fluidos, como la saliva, no permite una segura y directa introducción en el instrumento (determinaciones previas en nuestro laboratorio así lo han demostrado, resultados no publicados). Los principales métodos de preparación son: a) dilución; b) disolución y c) descomposición/digestión.

Una vez que el método se ha desarrollado, debe validarse, para asegurar que los datos derivados de su ejecución están de acuerdo con el "valor verdadero" del metal en las muestras. Esto puede realizarse por la determinación de los elementos en cuestión en un material de referencia, comprobando así que los resultados obtenidos son exactos y seguros. Son por tanto necesarios los materiales certificados de referencia (CRMs), los materiales estándar de referencia (SRMs) o materiales de referencia (RFs) con valores certificados de numerosos metales en diferentes muestras: órganos, tejidos, fluidos biológicos. En el caso de no existencia de materiales de referencia, el método debe validarse frente a otro método ya instaurado de solvencia o referencia, o utilizando criterios científicos de validación (González y Herrador, 2007). En ese sentido, resulta totalmente necesario elaborar materiales certificados de referencia, preparados tanto en disoluciones de saliva artificial, NaCl, ácidos orgánicos, así como fluidos biológicos, que contengan los iones metálicos que puedan liberarse de los procesos de corrosión de los materiales dentarios (Mikulewicz y Chojnacka, 2011a), y también resulta necesario estandarizar procedimientos analíticos. Específicamente *in vivo*, se requieren estudios que permitan la determinación de la exposición a metales a partir de aplicaciones ortodóncicas en muestras no invasivas, sobre todo tras exposiciones a largo plazo, como faneras (pelo, uñas) (Mikulewicz y Chojnacka, 2010), habida cuenta de que la duración media de estos tratamientos suele ser de 1,5-2 años.

Tabla 3. Concentración de metales traza en fluidos de pacientes debido a la liberación de iones metálicos a partir de diferentes aparatologías ortodóncicas (adaptado de Mikulewicz y Chojnacka, 2010; Matusiewicz, 2014)

Referencias	**Material**	**Fluido o tejido analizado**	**Iones metálicos medidos**	**Concentración (µg /L)**		**Método de detección**	**Pretratamiento de la muestra**
				Experimental	**Control**		
Kerosuo y col., 1997	Quad helix Anclaje extraoral Aparatología fija (bandas y brackets)	Saliva	Cr, Ni	78-108 65-85	61 55	GF-AAS	0,5 ml de las muestras de saliva se digirieron con 0,15 ml de HCl conc. Centrifugación 3000g durante 2 min.
Kocadereli y col., 2000	Aparatología fija superior e inferior (bandas y brackets)	Saliva	Cr, Ni	29-800 7-332	54 53	AAS	0.5 mL de la muestra de saliva se diluyeron con 10 mL de H_2O_2
Agaoglu y col. 2001	Bandas Brackets Arcos	Saliva Suero	Cr Ni Cr Ni	0,53-1,53[a] 4,12-11,53[a] 6,16-10,98[a] 7,87-10,27	0,76[a] 4,45[a] 6,21[a] 8,36	GF-AAS	El suero se preparó centrifugando las muestras de sangre a 3000 rpm durante 10 min.
Meningaud y col. , 2001	Miniplacas de titanio (ASTM F67-89)	Tejidos blandos	Ti	0,09-2,33	-	ICP-OES	Las muestras de tejidos secos fueron digeridas en un recipiente de Teflon con 65% HNO_3 en horno microondas
Faccioni y col. 2003	Arcos Bandas Brackets	Células de mucosa oral	Co Ni	0,568[a] 2.521[a]	0.202[a] 0.725[a]	ICP-MS	1 ml de suspensión de células de mucosa bucal se

	Arcos						trató con HNO_3 (2ml, 0.5%) y se diluyó con agua
Eliades y col. , 2003	Brackets de acero inoxidable	Saliva	Cr Fe Ni	27 17 10	11 14 6	ICP-OES	10-15 ml de saliva se secaron y mineralizaron utilizando agua regia en un sistema cerrado
Fors y Pearsson, 2006	Arcos Brackets Bandas	Saliva	Ni	0,005-25,25 µg/g	0,004 µg/g	GF-AAS	Las muestras de saliva se diluyeron en H_2O_2; 0,7g de la saliva fueron acidificadas en HNO_3
Menezes y col., 2007	Aparatología ortodóncica	Orina	Ni	19,89	17,67	AAS	-
Petoumeno y col., 2008	Aparatología ortodóncica	Saliva	Ni	28-78[a]	34[a]	ICP-MS	-
Matos de Souza y col., 2008	Aparatología ortodóncica	Saliva	Cr Fe Ni	0,3-1,7 28-104 1,7-16	0,6 94 5,3	GF-AAS	-
Amini y col., 2008	Arcos de NiTi	Células de mucosa oral	Co Cr Ni	0,84 4,24 21,74[a]	0,44 3,46 12,26[a]	GF-AAS	Las muestras de mucosa se diluyeron en agua y fueron acidificadas en HNO_3 a 60º , 10 min
Matos de Souza y col., 2008	Brackets cementados	Saliva	Cr Fe Ni	0,29-1,72 28,31-103,58 1,69-16,01	-	GF-ETAAS	La muestra de saliva se preparó secándola en horno eléctrico a 150º ,15 min

Petoumeno y col., 2009	Brackets Bandas Arcos	Saliva	Ni	56-78	34	ICP-MS	Las muestras de saliva se secaron, fueron digeridas con 0,2 ml agua regia; posterior dilución con H_2O_2
Amini y col., 2012	Arcos de acero inoxidable	Saliva	Cr Ni	2,6 18,5	2,2 11,9	AAS	1ml de saliva se diluyó con 10 ml de H_2O_2

[a]Las diferencias entre los grupos fueron estadísticamente significativas
AAS: Atomic absorption spectrometry; Espectrometria de absorción atómica
GFAAS: Graphite furnace atomic absorption spectrometry; Espectrometria de absorción atómica horno de grafito

ICP-OES: Inductively coupled plasma-optical emission spectroscopy, Espectroscopia de emisión atómica de plasma acoplado
ICP-MS: Inductively coupled plasma-mass spectrometry, Espectrometría de Masas con Plasma Acoplado Inductivamente.

4. IMPLICACIONES TOXICOLÓGICAS DERIVADAS DE LA LIBERACIÓN DE METALES A PARTIR DE APARATOLOGÍA ORTODÓNCICA

Las posibles implicaciones para la salud humana que se pueden derivar de la liberación de iones metálicos a partir de las aplicaciones ortodóncicas constituye un tema de enorme interés científico. En general, la mayoría de estudios *in vivo* que han evaluado la liberación de iones metálicos a partir de dichas aplicaciones en fluidos biológicos concluyen que las concentraciones alcanzadas son inferiores a la ingesta dietética diaria de algunos de los elementos (Kerosuo y col., 1997; Kocadereli y col., 2000; Agaoglu y col., 2001;), tales como la Ingesta Diaria Tolerable (IDT) establecida para el Ni de 2,8 µg/kg peso corporal /día (EFSA, 2015; http://www.efsa.europa.eu/en/search/doc/4002.pdf); o la Ingesta máxima estimada para el Co (0,039 mg/day) (EGVM, 2003; http://cot.food.gov.uk/sites/default/files/cot/vitmin2003.pdf); o el límite superior de ingesta tolerable (UL) establecido para el Cu de 5 mg/día o para el Cr trivalente de 1 mg/día, establecidos estos últimos por el Comité Científico de la Alimentación de la Unión Europea (SCF, 2003) y por la Autoridad Europea de Seguridad Alimentaria (EFSA, 2006; http://www.efsa.europa.eu/en/ndatopics/docs/ndatolerableuil.pdf).

Sin embargo, no puede excluirse que concentraciones no tóxicas de estos elementos puedan ser suficientes para producir daños biológicos/toxicológicos en la mucosa oral. Wataha y Schmalz (2009) indican que la ingesta dietética diaria de un elemento no es una buena regla para evaluar la seguridad de un material, ya que por ejemplo no tiene en cuenta las concentraciones elevadas de elementos que pueden ocurrir alrededor del mismo. Además, la concentración que se requiere para que se produzca un efecto adverso local puede ser mucho más baja que las concentraciones para causar efectos sistémicos a través de la vía oral. De hecho, se ha comprobado que diversos cationes metálicos liberados a partir de estos

materiales pueden causar alteraciones biológicas importantes (síntesis ADN, actividad fosfatasa alcalina, etc.) a concentraciones no citotóxicas (Geurtsen, 2002). Ocasionalmente, la respuesta del paciente a esta liberación de elementos metálicos va a diferir dependiendo de la naturaleza y cantidad de dicha liberación. Por ejemplo, las respuestas alérgicas clásicas se caracterizan porque no son dosis dependiente, es decir, bajas dosis pueden producir inflamación a través de una reacción tóxica que puede causarse por activación del sistema inmune (Schmalz y col., 2000). La liberación de componentes metálicos puede desencadenar una reacción alérgica (Leite y Nell, 2004), de forma que los tratamientos en ortodoncia dan lugar a reacciones de hipersensibilidad (Kalimo y col., 2004), aspecto último al que le dedicamos un apartado. Además, los efectos mutagénicos y carcinogénicos no están relacionados con la dosis del xenobiótico en general.

4.1. Aspectos toxicológicos de los iones metálicos liberados de aplicaciones ortodóncicas

Los principales elementos implicados en los procesos de corrosión como ya se ha indicado son Ni, Cr y Fe a partir de aplicaciones de acero inoxidable, y Ti y Ni a partir de aleaciones NiTi. De entre ellos, Ni y Cr han recibido particular atención por sus propiedades alergénicas, especialmente Ni, y por sus efectos citotóxicos, mutagénicos y carcinogénicos, que se resumen a continuación.

4.1.1. Níquel

El Ni es esencial para la actividad catalítica de algunas plantas y enzimas bacterianos, pero sin embargo, no se han demostrado sus funciones bioquímicas en el hombre, por lo que no es un elemento esencial para los humanos. El Ni está presente de forma natural en suelos, agua, plantas y animales. En sus compuestos tiene normalmente el estado de oxidación +2, pero puede existir en los siguientes estados: 0, +1, +3, y +4. Algunas de sus sales ingeridas por vía oral causan efectos tóxicos en riñón, médula, pulmones y sistema mieloide en animales de

experimentación.

Los porcentajes de este elemento en los materiales metálicos empleados en ortodoncia varían entre un 8% (acero inoxidable) y mas del 50% (NiTi aleaciones) (Railly y Price, 2003). Los humanos sensibilizados al Ni por contacto dérmico y que tienen dermatitis alérgica por contacto (estimándose que puede ser hasta un 15% de las mujeres, pero frecuentemente no diagnosticadas) desarrollan eczema en las manos por exposición oral o dérmica a sales del elemento. Se ha demostrado que ingestas orales tan bajas como 500 µg/día (aproximadamente 8 µg/kg p.c./día) agravan los eczemas en manos en sujetos sensibilizados a Ni (ver apartado Ni-sensibilización)

La absorción de las sales de Ni es bastante elevada en estado de ayuno, pero se reduce de forma significativa en presencia de alimentos (leche, café, te, zumo de naranja). Los picos máximos plasmáticos se alcanzan de forma rápida en 1,5-2,5 h (15-20 µg/L) y disminuyen en los próximos 3-4 días. Se une a la albúmina, histidina y α_2-macroglobulina y se distribuye ampliamente en el organismo. Se excreta principalmente por orina, (51-82% de la dosis en 5 días), y en menor extensión por la bilis y el sudor. También se secreta en la leche humana, y se ha demostrado que atraviesa la barrera placentaria en animales de experimentación. El contenido total en el organismo humano se estima en 0,5 mg. Los niveles más elevados ocurren en pulmón, tiroides, adrenales, y riñón. En humanos no ocupacionalmente expuestos, la concentración de Ni en sangre total y suero se encuentra en el rango de 1-5 µg/L y en orina es inferior a 10 µg/L.

La toxicidad aguda por vía oral de los compuestos de Ni depende de su solubilidad. Las sales solubles, cloruro y sulfato de Ni tiene valores de Dosis Letal 50 (DL_{50}) en ratas equivalentes a 42-129 mg Ni/kg peso corporal (pc). En humanos, los efectos no carcinógenicos tras exposición oral al Ni incluyen efectos gastrointestinales (GI), hematológicos, neurológicos y sobre el sistema inmune (EFSA, 2015). La exposición a través de la piel o por vía inhalatoria puede conducir a una reacción de sensibilización al Ni. Mientras que la exposición oral

al Ni no se conoce aún que conduzca a sensibilización, la absorción de Ni es capaz de inducir reacciones eczematosas en la piel de individuos sensibilizados al mismo (EFSA, 2015).

En relación con los estudios de toxicidad subcrónica, la administración por sonda de 25 mg sulfato Ni (kg/día) equivalentes a 9,5 mg Ni/kg/día durante 120 días, produjo severas lesiones en las células germinales, observándose espermiogénesis. En diversos estudios de dosis repetidas en ratas con sales de Ni (carbonato, cloruro, sulfato), los principales efectos observados han sido: disminución del peso corporal, disminución de hemoglobina, elevación de glucosa en suero, proliferación de células linfoides y micronecrosis en intestino a las dosis más elevadas (EFSA, 2006). En ratas, la administración repetida por vía oral de sulfato de Ni condujo a una disminución en el peso del hígado, del timo, y alteraciones en el sistema mieloide. Otros estudios demuestran que la administración de cloruro de Ni conduce a la producción de efectos tóxicos en riñón, hígado y médula, mientras que el sulfato de níquel hexahidratado ha producido daños en pulmón de rata.

Los estudios con animales de experimentación han demostrado que algunas sales de Ni (cloruro, sulfato) pueden producir alteraciones sobre la reproducción y desarrollo. El Panel de Contaminantes en la Cadena Alimentaria (CONTAM) de EFSA, ha identificado como efecto crítico para la caracterización del riesgo por exposición oral crónica al Ni, la toxicidad sobre la reproducción y el desarrollo, derivando recientemente la IDT de 2.8 μg Ni/kg peso corporal ya indicada (EFSA, 2015). En general, se considera necesario actualmente llevar a cabo estudios mecanísticos para evaluar la relevancia humana de estos efectos sobre la reproducción y desarrollo observados en animales de experimentación. En ausencia de datos adecuados dosis-respuesta para estos efectos, no es posible establecer un nivel de ingesta tolerable máximo (UL), según el Comité Científico de la Alimentación de la Unión Europea y la Autoridad Europea de Seguridad Alimentaria (EFSA) (EFSA, 2006; 2015).

La genotoxicidad del Ni y sus compuestos también ha sido revisada por diferentes organismos, incluyendo la Agencia Internacional de Investigaciones sobre el Cáncer (IARC, http://www.iarc.fr), la Organización Mundial de la Salud (OMS, WHO), la ATSDR (*Agency for Toxic Substances and Disease Registry,* http://www.atsdr.cdc.gov/es/) y la Comisión Europea (EC, 2004). Los estudios *in vitro* han demostrado que diversas sales solubles de Ni (sulfato, cloruro) producen aberraciones cromosómicas en células cultivadas de mamífero. También se ha demostrado la inducción de aberraciones cromosómicas en cultivos de linfocitos humanos con sulfato de Ni y sulfuro de Ni, y en linfocitos periféricos humanos el sulfato de Ni indujo micronúcleos (MN). Diversas sales de Ni (sulfato, cloruro) inducen roturas en el ADN, uniones cruzadas ADN-proteínas persistentes, inhibición de la síntesis y reparación del ADN. Asimismo también se ha demostrado que algunos compuestos de Ni inducen la transformación celular.

La interpretación de los resultados obtenidos en los estudios *in vivo* es más complicada. Algunas sales (sulfato, cloruro, nitrato) pueden inducir aberraciones cromosómicas en varias especies animales (rata, ratón), por diferentes vías (oral, intratraqueal o intraperitoneal (i.p.)). Los datos de los ensayos de MN son conflictivos, obteniéndose tanto resultados negativos como positivos. Algunos compuestos de Ni producen daño en el ADN, como roturas de las cadenas, uniones cruzadas ADN-proteínas, y el daño parece relacionado con procesos de inflamación y/o apoptosis celular. Los datos humanos sobre alteraciones cromosómicas son asimismo contradictorios.

Los mecanismos de acción genotóxica de los compuestos de Ni no están aún dilucidados. Estudios *in vivo* e *in vitro* han demostrado que producen roturas de las cadenas simples bien directamente o indirectamente. Se barajan diferentes mecanismos, entre los que cabe citar el daño oxidativo por generación de especies reactivas de oxígeno (ERO), o bien la inhibición de la síntesis del ADN ó la inhibición de los mecanismos de reparación del ADN, como se ha demostrado esto último *in vitro* a concentraciones no citotóxicas, siendo necesario profundizar

en dichos mecanismos (EFSA, 2006; 2015). La Comisión Europea (EC, 2004) ha clasificado en la categoría 3, Mutágenos, a las siguientes sales de Ni, sulfato, cloruro y nitrato.

La carcinogenicidad del Ni y sus compuestos ha sido evaluada por diferentes organismos internacionales, incluyendo la IARC y la Unión Europea (EC, 2004). Dicha agencia, evaluó globalmente a los compuestos de Ni en el grupo 1, de Carcinógenos humanos, basándose en las evidencias suficientes de los estudios epidemiológicos (cáncer de pulmón y nasal tras exposición por vía inhalatoria) y las evidencias suficientes en animales de experimentación. De acuerdo con la IARC, existe suficiente evidencia en animales de experimentación de la carcinogenicidad de implantes de Ni metálico y de aleaciones de Ni conteniendo aproximadamente 66-67% Ni, 13-16% Cr y 7% de Fe.

La Comisión Europea (Grupo de trabajo especializado en carcinogenicidad y mutagenicidad) concluyó que las siguientes sales de Ni: sulfato, cloruro, nitrato y carbonato se consideran carcinógenos humanos por inhalación (Categoría 1, frase R49: Puede causar cáncer por inhalación). No existen evidencias experimentales de producción de cáncer de los compuestos de Ni o Ni metálico tras exposición por vía oral, los datos disponibles hasta la actualidad son limitados. Existen algunas evidencias, de nuevo limitadas, que indican que los compuestos solubles de Ni pueden actuar como promotores, por vía oral (EFSA, 2006).

En el caso de aparatotología ortodóncica, la exposición a través de aleaciones que contengan Ni no son carcinógenas, y se considera que no deben existir riesgos, y de hecho no existen informes de carcinogenicidad asociada al uso intraoral de materiales que lo contengan (Wataha, 2000; Setcos y col., 2006).

Dedicamos un apartado especial a las reacciones de sensibilización inducidas por Ni registradas en la bibliografía científica.

4.1.1.1. Ni y sensibilización

Las sales de Ni son potentes sensibilizantes de la piel en humanos, que causan hipersensibilidad por contacto, relacionada directamente con la presencia del metal en el ambiente y causada por ingestión o contacto directo con la piel y/o mucosas (Volkman y col., 2007). Los iones Ni se unen a proteínas de la piel e inducen una respuesta inmunitaria celular retardada tipo IV, cuya prevalencia, características, etc. vemos a continuación. En individuos sensibilizados, no sólo la exposición dérmica, sino también la ingesta oral puede provocar eczema. Las dosis orales más bajas, administradas a sujetos sensibilizados que han provocado eczema han sido de 0,49 mg/día con un dieta alta en Ni (Nielsen y col., 1990), equivalentes a aproximadamente 8 µg Ni/kg p.c./día, y de 12 µg/kg p.c./día en el agua de bebida o con el estómago vacío. Las reacciones de hipersensibilidad al Ni en pacientes ortodóncicos han sido revisadas en la literatura científica (Bachman 1987; Hensten-Pettersen, 1989; Greppi y col., 1989; Kanerva y col., 1994; Lindsten y Kurol, 1997; Kerosuo y col., 1996; Janson y col., 1998; Noble y col., 2008).

Respuesta Inmunológica

De acuerdo con Coombs & Gell, existen cuatro tipos de reacciones alérgicas. Únicamente, dos de ellas son relevantes en el campo de la medicina dental y en la ortodoncia de forma más específica. La primera de ellas se trata de hipersensibilidad de tipo inmediata o, también denominada alergia de tipo humoral (Tipo I), la cual está desencadenada por Ig E específica y varios mediadores liberados predominantemente de los mastocitos. Este tipo de reacción se caracteriza por cursar con fiebre, asma bronquial y shock anafiláctico. El segundo tipo es la alergia mediada por células (Tipo IV), en ella se liberan linfoquinas procedentes de linfocitos T sensibilizados de forma específica (Schuster y col., 2004), y de este tipo es la dermatitis alérgica por contacto inducida por Ni. Se sugiere que los linfocitos T $CD4^+$ y $CD8^+$ están involucrados en la respuesta inmune al Ni (Noble y col., 2008). Las respuestas al Ni parecen

incluir la activación de linfocitos T específicos, seguido de la proliferación e inducción de diversas citoquinas incluyendo interferon IF-λ, así como interleucinas, IL-2, Il-5, IL-10, etc., estimulando la proliferación del tejido, lo que puede favorecer la hiperplasia gingival (Minang y col., 2005).

Epidemiología

Se estima que entre el 4,5-28,5% de la población presenta hipersensibilidad al Ni, siendo mas común en las mujeres (de 2-6 veces superior en comparación con los hombres), debido a una mayor exposición ambiental con el metal por el contacto con detergentes, bisutería diversa como uso de pendientes, y otros objetos metálicos, tales como botones etc. La alergia al Ni es mas común en pacientes mas jóvenes (Volkman y col., 2007). Se estima una tasa del 20% en el caso de mujeres en edades comprendidas entre 16-35 años, siendo menor, del 2%, en hombres debido al menor uso de bisutería (Noble y col., 2008). En los hombres la exposición es de tipo ocupacional. La sensibilidad al Ni posee una prevalencia mayor en personas asmáticas (Gül y col., 2007).

Los problemas de sensibilización y alergia al Ni son motivo de interés creciente en ortodoncia, y de controversia. La frecuencia de tratamientos de ortodoncia y el uso común del Ni en materiales de ortodoncia nos hace cuestionarnos si estos tratamientos pueden actuar incrementando o disminuyendo la hipersensibilidad al Ni en la población, especialmente tras constatarse un aumento de la prevalencia de los casos de hipersensibilidad por exposición a bisutería, piercing oral, etc., y otros materiales que contienen Ni.

Muchas aplicaciones auxiliares y utensilios empleados en ortodoncia contienen Ni, pudiendo variar entre un 8% (aceros por ej.) a más del 50% (aleaciones de Ni-Ti). Incluso los supuestos arcos libres de Ni como Menzanium^{R}, Noninium^{R} contienen cantidades traza de Ni (Schuster y col., 2004). Aunque el acero inoxidable contiene un 8% de Ni, esta aleación se considera segura para su colocación en pacientes alérgicos al Ni debido a su estructura cristalina enrejada y

a que la liberación es mínima (Rahilly y Price, 2003), aunque su relevancia clínica en pacientes alérgicos al Ni aún no esta clara.

Son diversos los casos reportados de hipersensibilidad al Ni ligadas a tratamientos en ortodoncia, y los factores que pueden influir (Noble y col., 2008; Kolokitha y Chatzistavrou, 2009). El contacto a largo plazo, el área de la piel implicada y su integridad son cruciales en el fenómeno de sensibilización. Los tratamientos de ortodoncia activos en niños y adolescentes generalmente duran de 2-3 años, y no sólo la aparatología intraoral, sino también las extraorales de Ni, pueden estar en contacto durante tiempo prolongado con las mucosas y la piel (Schuster y col., 2004). En general, aunque la presencia de iones metálicos como el Ni se asocia con reacciones de hipersensibilidad, en el caso de las aplicaciones de ortodoncia, la relevancia clínica es baja, existiendo casos muy contados.

Dada la controversia existente, Volkman y col., (2007) intentaron caracterizar la relación existente entre alergia al Ni y aplicaciones en ortodoncia sobre una población de más de 33000 pacientes, y sugirieron una prevalencia del 0,03%. Los resultados de este estudio fueron similares al llevado a cabo por Schuster y col. (2004), estimándose una prevalencia del 0,23%. Los pacientes presentaban signos de reacción alérgica tanto intraoral como extraoralmente. Por tanto, ambos estudios, coinciden en que la prevalencia de reacciones adversas a aplicaciones en ortodoncia conteniendo Ni es < 1%, baja si se compara con la prevalencia del 10-15% de dermatitis alérgica por contacto establecida para el Ni. Ello puede explicarse por (Rietschel y Fowler, 2001; Schuster y col., 2004; Volkman y col., 2007):

1) dilución del Ni en la saliva
2) la saliva puede formar una barrera disminuyendo la sensibilización
3) los anticuerpos IgA pueden actuar neutralizando alérgenos
4) la mucosa oral no queratinizada, carece de la capa de queratina donde las proteínas pueden combinarse con sustancias químicas para formar alérgenos
5) la mucosa oral contiene menos células de Langerhans, que juegan un papel crucial en la sensibilización de las dermatitis por contacto
6) la extensa vascularización de la mucosa oral previene el contacto prolongado con alérgenos por absorción y dispersión de los mismos

Una revisión acerca de los síntomas, diagnóstico, tratamiento de los casos informados de alergia motivados por materiales empleados en ortodoncia ha sido llevada a cabo por Noble ty col., (2008) y se considera que es necesaria una mayor concentración de Ni en la mucosa oral que en la piel para desencadenar una reacción alérgica. Como el nivel de tolerancia de la mucosa oral es superior a la de la piel queratinizada, se confirma con estos trabajos la validez de la práctica en ortodoncia de ensayar la compatibilidad individual de forma gradual, empleando inicialmente sólo un bracket o fijando solo una banda. De forma que si no se registran reacciones alérgicas potenciales, se puede continuar el tratamiento con materiales con Ni. En definitiva, parece que las condiciones, estructura y la inmunología de la cavidad oral son diferentes a las de la piel, y ello hace que sea menos reactiva en boca tras el contacto con alérgenos como el Ni. Otros estudios, sin embargo, elevan las tasas de reacciones alérgicas al Ni en tratamientos de ortodoncia, llegando incluso la prevalencia a ser del 17,2% tras 9 meses de tratamiento (Pazzini y col., 2009).

Janson y col. (1998) determinaron la prevalencia de reacciones de hipersensibilidad antes, durante y después de un tratamiento de ortodoncia con brackets y arcos convencionales de acero inoxidable. Emplearon el ensayo de parche al Ni y un cuestionario para evaluar la hipersensibilidad en un total de 170 pacientes, divididos en tres grupos: pacientes antes del comienzo del tratamiento de ortodoncia, pacientes que se encuentran en tratamiento, y por último pacientes que han tenido tratamiento previo (entre 2 meses y 6 años después). Demostraron una tasa elevada de reacciones alérgicas, del 28,3%, superior en mujeres (23%) que en varones (5,3%), existiendo por tanto diferencias ligadas al sexo. También demostraron una asociación positiva entre hipersensibilidad al Ni e historial previo de alergia al Ni, así como con el uso diario de objetos metálicos, evidenciando una manifestación temprana de dicha reacción en personas con hipersensibilidad al metal, corroborando estudios anteriores (Blanco-Dalmau y

col., 1984; Lamster y col., 1987; Dunlap y col., 1989). Al no encontrar diferencias significativas entre los 3 grupos ensayados, sugieren que la terapia ortodóncica no inicia o agrava una reacción de hipersensibilidad al Ni. Otras variables, como historial familiar de alergia, grupo sanguíneo, presencia de restauraciones y su número, tiempo transcurrido entre eliminación de la aplicación, no caracterizaron la hipersensibilidad al Ni estudiada (Janson y col., 1998).

Diversos estudios indican que la sensibilidad al Ni parece menor en sujetos que han recibido tratamiento ortodóncico, quizás porque desarrollen tolerancia inmunológica tras un periodo largo de tratamiento (Van Hoogstraten y col., 1991y 1993; Kerosuo y col., 1996; Artik y col., 2001; Mortz y col., 2002), indicándose que un contacto temprano con alérgenos potenciales conlleva a que la probabilidad de reacciones alérgicas posteriores, a lo largo de la vida, sea menor (Van Hoogstraten y col., 1991). En ortodoncia, en relación con el concepto de tolerancia, existen estudios que indican que el tratamiento con aplicaciones que contienen Ni previos a la sensibilización al Ni disminuye la incidencia de hipersensibilidad al Ni (Greppi y col., 1989; Bass y col., 1993), relacionándose dicha tolerancia con la lenta liberación a largo plazo de los aparatos ortodóncicos (Hensten-Pettersen, 1989). Otros autores concluyen que las aplicaciones en ortodoncia no inducen ni tolerancia ni reacciones de sensibilización (Menezes y col., 2004). De forma general, se considera que una historia previa de alergia debe considerarse como un factor predictivo de las manifestaciones clínicas de la hipersensibilidad al Ni (Genelhu y col., 2005) y el tiempo de colocación del aparato ortodóncico es realmente importante.

· Diagnóstico

El diagnóstico de una reacción de sensibilización de la mucosa oral al Ni es más difícil, en comparación con la piel. Se debe determinar una alergia previa a través de cuestionarios médicos apropiados, o durante la revisión oral del historial médico, de forma que se debe alertar al paciente de una posible respuesta al Ni, particularmente cuando se instala el arco inicial. Si se cuestiona una posible

reacción se puede confirmar mediante un ensayo de sensibilidad cutáneo empleando sulfato de Ni 5% en petróleo (Janson y col., 1998). El ensayo del parche cutáneo, originalmente desarrollado y descrito por Jadassohn, es el método más empleado en materiales dentales para identificar reacciones de hipersensibilidad tipo IV (Schmalz, 2009). El diagnóstico de una reacción de hipersensibilidad tipo IV es posible sólo si los signos clínicos de la alergia se asocian con la presencia de un ensayo positivo (ensayo del parche) del alérgeno presente en la cavidad oral (Schmalz, 2009). Estudios con pacientes sujetos al ensayo del parche, han mostrado frecuentemente reacciones positivas a metales, como Au y Ni. Este ensayo ha sido aplicado por Pazzini y col., (2009), de forma que todos los pacientes que fueron considerados negativos no presentaron signos clínicos visibles, y todos los pacientes positivos (Figura 3) presentaron eritema, edema, pápulas y ampollas.

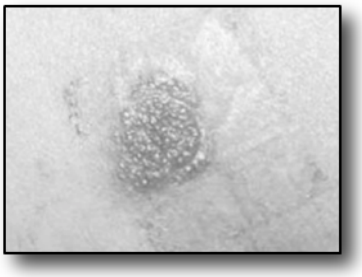

Figura 3. Reacción positiva al ensayo
(Pazzini y col., 2009)

La principal indicación para realizar un ensayo epicutáneo es la presencia de síntomas intraorales próximos a una aplicación ortodóncica; también, como se ha comentado, se han referido casos severos de descamaciones intraorales generalizadas o erosiones yo eczema perioral, en las que se requiere su evaluación alérgica. Por otro lado, un resultado negativo no es garantía de ausencia futura de hipersensibilidad (Wataha y Schmalz, 2009; Arenholt-Bindslev y col., 2009).

· Signos y Síntomas:

Entre los **signos y síntomas clínicos** orales se destacan: sensación de quemazón, hiperplasia gingival, descamación labial, quelitis angular, eritema

multiforme, periodontitis, estomatitis con eritema medio o severo, rash papular perioral, pérdida del sabor o sabor metálico, adormecimiento y dolor en el lateral de la lengua, como podemos observar en la Figura 4 (Eliades y Athanansios, 2002).

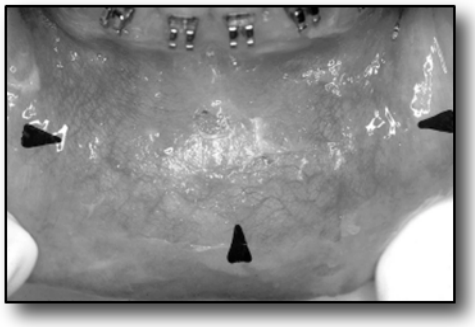

Figura 4. Inflamación eritematosa de la región anterior del labio inferior tras el cementado de brackets de acero inoxidable y la inserción de un arco de NiTi (Eliades y Athanasios, 2002)

Se pueden producir reacciones eczemáticas y urticaria sobre la cara o en zonas de la piel más distantes (Kolokitha y Chatzistavrou, 2009). Diversos autores añaden dermatitis sistémica de contacto, así como localizada tanto intraoral como extraoral, y conjuntivitis debido a brackets, bandas y retenedores (Mancuso y Berdondini, 2002; de Silva y col. 2000; Pigatto y Guzzi, 2004;) En la siguiente Figura se evidencian estas reacciones extraorales en región perioral (Schmalz, 2009).

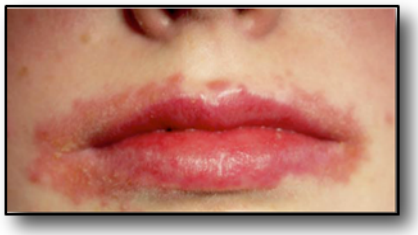

Figura 5. Manifestaciones extraorales de reacción alérgica al Ni (Schmalz, 2009).

Se puede afirmar que todas las manifestaciones extraorales de alergia al Ni tienen su origen intraoralmente (Schultz y col. 2004). Además de las alteraciones

a nivel de la piel, las cuales son difíciles de diagnosticar, los pacientes mencionan síntomas generales, como dolor de cabeza, migraña, mialgia y humor depresivo, expresándolos como síntomas de la sensibilización alérgica (Regland y col., 2001).

En relación con el Tratamiento, existen diferentes materiales disponibles como alternativas, recogidas en la Tabla 4 (Eliades y Athanasios, 2002)

Categoría	Material	Sustitutos libres de Ni y Modificaciones
Aparatología estándar	Brackets	Brackets de acero inoxidable, cerámicos, plástico, Ti, Au y otros metales preciosos libres de Ni
	Bandas	Bandas de Au
Utensilios de tratamiento	Arcos de acero inoxidable	No existen arcos alternativos; el desarrollo de arcos de polímeros está en proceso
	Arcos de Níquel-Titanio (NiTi)	Arcos de β-Ti (TMA), nitride- or epoxy-coated NiTi y de ∝-Ti
	Arcos de CoCrNi (Elgiloy)	No existen alternativas
Auxiliares mecánicos	Arco lingual Barra transpalatina Sliding yokes	Segmentos de arcos de β-Ti (TMA), plástico, o cubiertas de metal inerte (oro)
Auxiliares misceláneos	Ligaduras de acero inoxidable	Ligaduras cubiertas de Teflon
	Hooks Kobayashi	Hooks Kobayashi de Teflon; brackets sin Ni con hooks
	Coil springs	Ligaduras elastoméricas
Aparatología fija de expansión	Quad-helix, Disyuntores	Arcos de β-Ti (TMA) para el QH
	Anclaje extraoral	Arcos extraorales cubiertos de Teflon
	Minitornillos de NiTi	No existen alternativas
Aparatología removible	Componentes de acero inoxidable de la placa de Hawley y variaciones	Retenedores de plástico o elásticos; posicionadores elásticos

Categoría	Material	Sustitutos libres de Ni y Modificaciones
Intervenciones terapéuticas complejas	Placas y tornillos de cirugía ortognática Aparatos de distracción osteogénica	Placas y tornillos de material poliacrílico-poliglicólico reabsorbible No existen alternativas

Tabla 4. Alternativas de tratamiento para aquellos pacientes con alergia al Ni (Eliades y Athanasios, 2002).

Si el paciente tiene un historial previo de alergia, se deben evitar los tratamientos con Ni, y en su lugar se aconsejan como tratamientos alternativos el empleo de brackets cerámicos (alúmina policristalina, sapphire single-crystal sapphire, y Zr), de policarbonato, de Ti o de Au-plated. Ya hemos comentado que en relación con los arcos, el acero inoxidable es un material considerado como seguro a emplear en componentes intraorales en pacientes sensibilizados. Se debe eliminar el arco Ni-Ti, reemplazarlo por un arco de acero inoxidable (con un contenido menor en Ni) y preferiblemente por aleaciones Ti-Mo (TMA) los cuales no contienen Ni, o bien, por arcos de NiTi recubiertos de resina (Wataha y col., 1999).

Si el paciente continuara manifestando la reacción alérgica, se deben eliminar todos los arcos, brackets de acero, tratar al paciente con antihistamínicos, anestésicos o corticoides tópicos. La administración de sustancias con corticoides para contrarrestar la hipersensibilidad afecta al proceso de movimiento ortodóncico, reduciendo la tasa de movimiento, por lo que la administración debe evitarse si los signos no son severos (Melsen, 1978; Bachmann, 1987; Greppi y col., 1989; Janson y col., 1998).

4.1.2. Cromo

Las aplicaciones ortodóncicas pueden contener por término medio un 17-22% de Cr, y este elemento suscita gran interés por su potencial alergénico, así como por sus propiedades citotóxicas y mutagénicas, incluyendo carcinógenicas,

en el caso de Cr hexavalente (VI). El Cr en una aleación, puede incrementar las propiedades de resistencia a la corrosión. Se añade a las aleaciones basadas en el Ni para mejorar sus habilidades con el fin de formar una película de óxido protectora en su superficie. Generalmente, la superficie de la aleación está formada por una capa de óxido de Cr. Se ha sugerido que un contenido del 16-27% de Cr proporciona una óptima resistencia a la corrosión en las aleaciones de Ni (Brune, 1986).

El Cromo (Cr) es un elemento ubicuo, presente en aguas, suelo y sistemas biológicos, que puede estar presente en diferentes estados de oxidación, desde Cr^{o} a Cr^{+6}. Las tres formas más estables son 0, +3, +6, que se corresponden con las formas de metal y aleaciones, Cr trivalente (III) y Cr (VI), respectivamente. La forma más usual presente en la naturaleza son los compuestos de Cr (III), siendo muy diferentes sus efectos biológicos y tóxicos en comparación con las formas de Cr (VI), como los cromatos, de mayor importancia industrial.

El Cr (III) está considerado como elemento esencial en animales y en la nutrición humana. Influye en el metabolismo de carbohidratos, lípidos y proteínas, a través de sus efectos sobre la insulina, aunque los mecanismos aún no están totalmente clarificados. Los estudios limitados existentes sobre la toxicidad subcrónica, crónica y efectos tóxicos sobre la reproducción de las sales de Cr (III), y los escasos datos humanos, no permiten obtener datos adecuados dosis-respuesta, por lo que no es posible establecer un nivel de ingesta tolerable máximo (EFSA, 2006). El grupo de Expertos en Vitaminas y Minerales de Reino unido (EGVM) ha considerado que una ingesta diaria de aproximadamente 0,15 mg de Cr (III)/kg peso corporal/día (10 mg/persona) se espera no produzca efectos adversos sobre la salud, exceptuando al compuesto picolinato de Cr, debido a que *in vitro* se ha demostrado que puede dañar el ADN por un mecanismo no totalmente dilucidado hasta la actualidad (EGVM, 2003).

Los compuestos de Cr (VI) son más tóxicos que los compuestos trivalentes, siendo corrosivos y causando úlceras crónicas y otras alteraciones en la piel,

independientes de la reacciones de hipersensibilidad (Goyer y Clarkson, 2001). Los efectos tóxicos del Cr en humanos se atribuyen a la forma hexavalente, y se ha especulado que los efectos biológicos de Cr (VI) están relacionados con la reducción a Cr(III) y la formación de complejos con macromoléculas intracelulares.

La absorción de Cr (III) depende, entre otros factores, de las propiedades químicas del compuesto ingerido, del nivel de ingesta a través de la dieta y de la presencia de diversos componentes en la misma, por la existencia de interacciones. El Cr se une a proteínas plasmáticas, como transferrina, afectando a la unión del Fe con dicha proteína. El Cr (VI) es tomado selectivamente por los eritrocitos, reducido a Cr (III) por el glutation (GSH) y se une a la hemoglobina. Por tanto, el Cr se encuentra solo en plasma tras absorción gastrointestinal de Cr (III), y entre plasma y eritrocitos en el caso de Cr (VI).

Sólo nos referimos a la toxicidad oral de los compuestos de Cr, que ha sido revisada por diferentes organismos internacionales (ATSDR, 2012; EGVM, 2003).

La toxicidad aguda por vía oral de los compuestos de Cr varia en función del compuesto, oscilando las DL_{50} en rata y ratón de los compuestos solubles trivalentes entre 140 – 422 mg/kg (EGVM, 2002). Son muy escasos los estudios de toxicidad subcrónica publicados en la literatura científica. La administración de diferentes dosis de Cr_2O_3, a través de la dieta no produjo efectos tóxicos, ni carcinógenos, probablemente por la pobre absorción del compuesto. En otro experimento similar, sólo se detectó una ligera disminución del peso del hígado y bazo, con la dosis más elevada.

Con respecto, al **Cr y Reacciones Alérgicas,** los compuestos de Cr pueden dar reacciones alérgicas en la piel tras exposición, y de forma independiente de la dosis. Las dermatitis alérgicas por contacto causadas por sales de cromatos fueron notificadas por primera vez en 1925, y es un fenómeno aún común. Los compuestos de Cr (VI) están considerados como los sensibilizantes de Cr más

potentes. De acuerdo con los ensayos de maximización, pertenecen a la categoría de sensibilizantes de fuertes a extremos. Se acepta generalmente que el Cr metal no actúa como un hapteno y no es sensibilizante. Teóricamente, fluidos como el sudor y el plasma pueden transformar los compuestos metálicos de Cr en sales de cromato alergénicas. La saliva puede tener un efecto similar sobre los aparatos intraorales que contienen Cr. La alergia por contacto tiene alta incidencia en trabajadores de la construcción por la posible presencia de Cr (VI) soluble en algunos cementos; también en la industria del curtido de la piel, etc.

A menudo no está claro si los cromatos o bien otros metales y sus sales son los agentes causales de reacciones alérgicas provocadas por aleaciones dentales. En muchos casos en los que la reacción alérgica se ha atribuido al Cr, el sensibilizante real ha sido el Ni. Se ha registrado un caso de dermatitis en las manos en un paciente alérgico al Cr, y negativo al Ni en el ensayo del parche cutáneo. En la Figura 6 se muestra la dermatitis vesicular en una chica de 15 años, tras la inserción de aplicaciones ortodóncicas. No se observó estomatitis, y el ensayo del parche fue positivo al cromato (Arenholt-Bindsley y col., 2009).

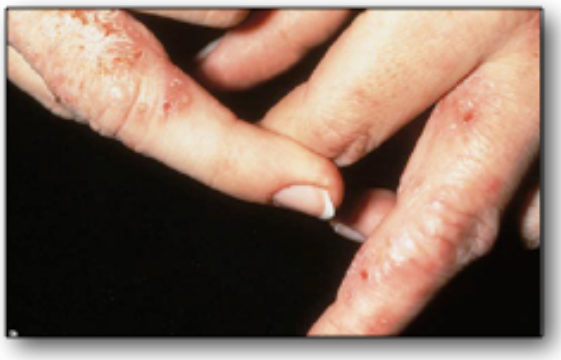

Figura 6. Dermatitis vesicular tras colocación de aparatología ortodóncica (Arenholt-Bindslev y col., 2009).

La genotoxicidad de un gran número de compuestos de Cr se ha ensayado *in vitro* e *in vivo*, estudios que se han sido revisados por diferentes organismos (EFSA, 2006; IARC). Al evaluar los resultados es necesario tener en cuenta varias

propiedades de los compuestos en cuestión, como estado de oxidación, solubilidad, capacidad para atravesar membranas, estabilidad intracelular, y reactividad con componentes celulares, etc. Se ha demostrado que diversos compuestos de Cr (VI) son genotóxicos, y los compuestos de Cr (III) aunque son más reactivos con los ácidos nucleicos purificados produciendo uniones cruzadas ADN-proteínas, generalmente no producen mutaciones de genes, intercambio de cromátidas hermanas o transformaciones celulares en cultivos de células de mamíferos. No se ha observado la inducción de daño genético o de micronúcleos (MN) en animales de experimentación. Se ha demostrado que Cr (III) (cloruro) y Cr (VI) (dicromato potásico) dieron resultados positivos en el ensayo cometa realizado en linfocitos periféricos humanos aislados. Se sugiere que las especies reactivas de oxígeno (ERO), y peróxido de hidrógeno pueden estar involucradas en la formación de roturas de bandas del ADN por Cr (VI), pero no por Cr (III).

En humanos, Medeiros y col., (2003) estudiaron los efectos de compuestos de Cr (III) en curtidores expuestos de forma crónica, y los de Cr (VI) en soldadores manuales de arcos metálicos de acero inoxidable, utilizando como marcadores la formación de uniones cruzadas ADN-proteínas (DPC) y la presencia de MN en linfocitos periféricos. Los resultados indicaron una relación causal entre exposición a compuestos de Cr en ambos casos y niveles de DPC en linfocitos, siendo mayor en el caso de los soldadores. En los curtidores también se observó un incremento de MN en linfocitos, siendo además su interpretación compleja.

Un estudio ha revelado que el cloruro de Cr (III) disuelto en el agua de bebida, en ratones macho y hembra, durante 1 semana, produjo una disminución de la fertilidad de forma significativa, estimándose que las concentraciones de Cr (III) oscilaron aproximadamente entre 250-1250 mg/kg peso corporal/día. No existen estudios publicados acerca de la toxicidad sobre el desarrollo de los compuestos de Cr (III) por vía oral (EFSA, 2006).

La carcinogenicidad del Cr (III) ha sido evaluada por la IARC, y tras una

revisión de los diferentes estudios experimentales con animales (rata, ratón), y por diferentes vías, concluye que existen "evidencias limitadas en animales de experimentación de la carcinogenicidad de Cr_2O_3 y de dicromato sódico", así como "evidencias inadecuadas de la carcinogenicidad de Cr metal, cromato de bario y los compuestos de Cr (III)". En cuanto a los datos humanos, la IARC concluye que "Cr metálico y los compuestos de Cr(III) no son clasificables por su carcinogenicidad en humanos, grupo 3". Las especies de Cr (VI) están ampliamente consideradas como agente responsable de un mayor riesgo de cáncer de pulmón en trabajadores del Cr (producción de dicromato, pigmentos de cromato, aleaciones de cromo), estando clasificadas en el grupo 1 (suficiente evidencia en humanos) (IARC, http://monographs.iarc.fr/ENG/Monographs/vol100C/mono100C-9.pdf), pero no existen datos adecuados en relación con los compuestos de Cr (III) (IARC), por lo que están clasificados en el grupo 3 (http://monographs.iarc.fr/ENG/Monographs/suppl7/Suppl7-51.pdf).

4.1.3. Cobre

El Cu se puede liberar a partir de aplicaciones ortodóncicas (bandas) y su exceso está implicado en la formación de radicales $OH^{\cdot}$ que puede iniciar reacciones de peroxidación lipídica no específicas (Gonçalves y col., 2014).

Es un elemento traza esencial, siendo un componente imprescindible de muchos enzimas (cuproenzimas) y proteínas. Su papel es primariamente catalítico, ya que muchas metaloenzimas que lo contienen actúan como oxidasas, tales como citocromo-C-oxidasa y superoxido dismutasa (SOD), esencial en mecanismos de estrés oxidativo. Se requiere Cu para el crecimiento infantil, para los mecanismos de defensa, maduración de los glóbulos rojos y blancos, transporte de Fe, metabolismo de la glucosa y colesterol. También juega un papel adicional menos conocido en la angiogénesis, mielinización de los nervios y acción de endorfinas. Se ha estimado una ingesta segura y adecuada a través de la

dieta, entre 1,5-3,0 mg/día. Aunque los niveles de Cu están sometidos a control homeostático, de forma que ante la ingestión de un exceso, se reduce su absorción y se incrementa su excreción, se han dado intoxicaciones tanto agudas como crónicas (EFSA, 2006)

La intoxicación aguda por Cu en humanos es poco frecuente, y los síntomas incluyen salivación, dolor epigástrico, náuseas, vómitos y diarrea. Las intoxicaciones crónicas en humanos han sido poco estudiadas, existiendo datos que sugieren que la exposición crónica a Cu causa diarreas e irritación GI.

Aunque se ha demostrado que induce lesiones en el ADN en hígado de pacientes con enfermedad de Wilson, no existen evidencias de que produzca cáncer; tampoco existen evidencias de que su exceso esté ligado a una mayor incidencia de enfermedades coronarias. Parece que juega un papel importante en enfermedades neurológicas, y se especula que la producción de radicales hidroxi puede contribuir a la neurodegeneración de la enfermedad de Alzheimer's. La enfermedad de Wilson se caracteriza por la acumulación excesiva de Cu en hígado, cerebro, riñones y córneas, y ocurre por anomalías en la excreción biliar del elemento, observándose en sangre un descenso de la ceruloplasmina y elevación del Cu libre (Goyer y Clarkson, 2005). Es posible que las personas con déficit de glucosa-6-fosfato sean más vulnerables a los efectos hematológicos del Cu. En humanos, no existen evidencias que relacionen la ingesta oral de Cu y toxicidad sobre la reproducción (EFSA, 2006).

El Cu posee también propiedades citotóxicas y puede inducir apoptosis (Geurtsen, 2000; Rana, 2008).

4.1.4. Cobalto

El Co puede también producir alergias, asociado con Ni y cromato, de forma que se puede tratar de una sensibilización concomitante, ya que suele estar presente en los productos de Ni y Cr. En la mayoría de los casos, la alergia ocurre en asociación con sensibilidad al Ni en mujeres, y al Cr en hombres.

Las sales de Co se absorben generalmente bien tras ingestión oral, y aproximadamente el 80% se excreta por orina. La administración oral crónica de altas dosis de Co para el tratamiento de anemias puede conducir a la producción de bocio, y un exceso del metal puede producir vómitos, diarrea, y cardiomiopatías. Es débil mutágeno y no existen evidencias de sus carcinogenicidad por diferentes vías de exposición (Goyer y Clarkson, 2001).

Cobalto metal y sus iones son citotóxicos e inducen apoptosis, y a elevadas concentraciones necrosis con respuesta inflamatoria (Simonsen y col., 2012). Es conocido que es cardiotóxico en animales y en humanos. En el hombre, por ej., se han descrito casos de cardiomiopatías en fuertes consumidores de cerveza (años 60s) por contener cloruro de cobalto como aditivo, llegándose a alcanzar ingestas de 6.8 mg Co/día. Pocos son los datos de toxicidad que existen en humanos. Los casos reportados sugieren que unas ingestas agudas de 30 mg/día de Co pueden causar malestar GI, rash cutáneos y fiebre. Ingestas crónicas de Co entre 0.17 – 0.39 mg/kg (10,2 – 23,4 mg total en un adulto de 60 kg peso) pueden deprimir la absorción de yodo. Sus mecanismos de toxicidad aún no están dilucidados, y los datos son insuficientes para establecer una relación dosis-respuesta, por lo que no se existe aún un Nivel superior seguro de ingesta de este elemento (EGVM, 2003).

4.1.5. Hierro

El Fe es un elemento esencial que tiene importantes funciones metabólicas, incluyendo el transporte y almacenamiento de O_2 (hemoglobina y mioglobina), y participa en forma hemo en numerosas reacciones redox de citocromos. También hay que considerar los aspectos toxicológicos, que son importantes en términos de su deficiencia (anemia, disminución de la función inmune, etc.) existiendo diversos grupos vulnerables a dicha deficiencia, así como por intoxicaciones tras exposiciones agudas accidentales, y sobrecarga crónica debida a hemocromatosis idiopática o como consecuencia de un exceso de Fe en la dieta.

Su disponibilidad está regulada por un complejo mecanismo para mantener la homeostasis. Por regla general, en el tubo digestivo se absorbe entre el 2-15% del Fe, mientras que la eliminación supone únicamente cerca del 0,01% al día (Goyer y Clarkson 2005). El Fe absorbido se une a la transferrina plasmática, que los transporta hacia sus depósitos en la hemoglobina, mioglobina, las enzimas que contienen Fe y las proteínas de depósito del Fe, como la ferritina y la hemosiderina. En condiciones normales, el exceso de Fe ingerido se excreta.

La intoxicación aguda por Fe suele ser secundaria a la ingestión, accidental o intencionada, de medicamentos que lo contienen, observándose fundamentalmente efectos GI, hepáticos, pancreáticos y cardiovasculares. También se presentan efectos adversos GI (náuseas, dolor epigástrico, constipación) por consumo a corto plazo de suplementos de Fe no hemo (50-60 mg/día).

Como se ha indicado, la sobrecarga de Fe con síntomas clínicos (disfunción hepática, incluyendo cirrosis hepática) puede tener tres causas principales: 1) aporte excesivo de Fe en la dieta o tratamiento médico con altas dosis de Fe (160-1200 mg/día); 2) individuos con hemocromatosis hereditaria (homocigotos) incluso con niveles normales de Fe en la dieta y 3) transfusiones de sangre frecuentes en algunas anemias resistentes al tratamiento.

Diversos estudios epidemiológicos han encontrado asociaciones entre alta ingesta y/o almacenamientos de Fe y riesgo incrementado de enfermedades crónicas, tales como enfermedades cardiovasculares, diabetes tipo II y cáncer del tracto GI, aunque los datos son contradictorios y no existen evidencias concluyentes sobre la relación causa-efecto. El panel de Productos dietéticos, Nutrición y Alergias de EFSA ha considerado que los datos disponibles son insuficientes para establecer una nivel máximo de ingesta tolerable para el Fe (EFSA, 2006).

Los mecanismos exactos por los que el Fe induce estas alteraciones no están totalmente dilucidados, aunque la toxicidad última parece resultar de su papel

catalítico en la generación de radicales libres a través de la reacción tipo Fenton, conduciendo al daño oxidativo de biomoléculas de todo tipo (Sochaski y col., 2002). En la relación Fe - estrés oxidativo, indicar que el ión Ferroso puede incrementarlo, porque las ERO pueden convertir aniones superóxido en radicales hidroxilos altamente reactivos a través de la conocida reacción de Fenton, catalizada por Fe (McCord and Turrens, 1994).

$$Fe\ (II) + H_2O_2 \rightarrow Fe(III) + {}^{\cdot}OH + OH^-$$

Los radicales hidroxilo son capaces de reaccionar de forma indiscriminada con macromoléculas celulares, originando degradación de proteínas, inactivación de enzimas, LPO, daño en el ADN y, en última instancia, muerte celular.

De hecho, experimentalmente, se ha demostrado que la sobrecarga crónica de Fe induce un aumento de LPO de forma dosis-dependiente, aumentando de forma concomitante los aldehídos reactivos en plasma, modificándose las proteínas de los tejidos, y disminuyendo la actividad de la enzima glutatión peroxidasa (GPx) en ratón, demostrándose por tanto, la participación del estrés oxidativo como mecanismo de acción de las alteraciones patológicas crónicas producidas por el elemento (Sochaski y col., 2002). El exceso de Fe, al contribuir con una mayor LPO, produce consiguientemente afectación de la membrana de las mitocondrias, los microsomas y otros orgánulos celulares (Goyer y Clarkson, 2005).

4.2. Citotoxicidad de aparatología ortodóncica

Los ensayos de citotoxicidad constituyen la primera etapa para estudiar la biocompatibilidad de material ortodóncico y reducir así el empleo de animales de laboratorio (Assad y col., 1994).

Está bien documentado que algunas aleaciones de materiales empleados en ortodoncia son citotóxicas *in vitro*, y que el daño puede correlacionarse con la liberación de elementos a partir de estos materiales. Sin embargo, las correlaciones entre liberación de iones metálicos y citotoxicidad son

extremadamente complejas, de forma que la liberación de iones metálicos (con propiedades citotóxicas como se ha documentado anteriormente), es necesaria para causar daño celular, pero a veces no es suficiente (Wataha y col., 2009). Dicho de otro modo, hace falta que un elemento sea liberado para que se manifieste citotoxicidad, aunque no todos los elementos liberados provocan un efecto citotóxico. La citotoxicidad de una aleación en general depende del tipo de elemento liberado, de su concentración y, probablemente, de su forma. Por ejemplo, la aleación Ag-Pd libera Ag, Cu y algo de Pd en el medio, pero la aleación no presenta características citotóxicas. La aleación de Oro-Cadmio libera menos Ag y Cu y no libera Pd, pero también libera Cd. En segundo lugar, la tendencia de un elemento a ser liberado de una aleación (labilidad) nos dice poco de su capacidad de producir un efecto tóxico en la célula. Elementos como el Au y el Pd, pueden ser tóxicos sólo en concentraciones relativamente moderadas a altas y tener una labilidad baja a muy baja en la aleación. Por ello, estos elementos representan un riesgo bajo desde el punto de vista biológico. Elementos como el Cu, presentan una toxicidad débil a moderada, pero tienen una alta labilidad en la aleación. Por ello, el riesgo de emplear estos elementos es relativamente más alto. Por último, elementos tales como el Cd tienen una alta toxicidad y alta labilidad, por lo tanto, representan el riesgo más alto biológicamente. Es importante resaltar que no basta con ver si un elemento está presente en la composición de la aleación, sino que también hay que verificar si está liberado y si es tóxico en las concentraciones emitidas. La labilidad de un elemento puede verse influenciada por la presencia de otro elemento. Por ejemplo, el Pd en concentraciones suficientes parece reducir la labilidad de Cu en la mayoría de las aleaciones (Wataha y col. 1991, 1992, 1995).

Los ensayos de citotoxicidad *in vitro* son frecuentes ya que se consideran un índice primario de biocompatibilidad (Kao y col., 2007). Son necesarios durante el desarrollo de un material, y son rápidos y de bajo coste en comparación

con los ensayos *in vivo*, aunque la extrapolación de los resultados a los pacientes es a menudo cuestionable (Schmalz, 2009).

La calidad y especificad de los datos generados por los modelos *in vitro* dependen de los siguientes factores (Castell y col., 1997):

1. El empleo de un sistema biológico que reproduzca el comportamiento metabólico del órgano diana para el efecto tóxico del xenobiótico.
2. La elección de parámetros adecuados de evaluación del efecto tóxico *in vitro*
3. Un correcto diseño experimental, de forma que los datos *in vitro* sean predictivos de los potenciales efectos in vivo.

In vitro, las células se exponen a las disoluciones de iones liberados, extractos de los materiales, etc., y se pueden determinar diferentes parámetros, además de la viabilidad celular, como la síntesis de proteínas, actividades enzimáticas diversas, y mediadores inflamatorios. Uno de los ensayos más empleados para la determinación de citotoxicidad lo constituye la captación del colorante rojo neutro (RN), marcador de integridad lisosomal, que tiñe a las células vivas, mientras que las células dañadas no se tiñen. Otro ensayo frecuente es el ensayo del MTT (3,-[4,5-dimetiltiazol-2-il]2,5difeniltetrazolio bromuro] para determinar actividad mitocondrial. Metales como Cr, Ni y Co pueden causar apoptosis a concentraciones más bajas que las que producen necrosis celular, por lo que son usuales los estudios morfológicos de las células expuestas. Y a nivel molecular, para estudios de mecanismos de acción tóxica, se pueden determinar especies reactivas de oxígeno (ERO), apoptosis, daño y reparación del ADN, cambios o síntesis de mediadores específicos de la inflamación, etc.

En la bibliografía consultada, son diversos los estudios que evalúan *in vitro* la liberación de metales a partir de aplicaciones en ortodoncia, como brackets y arcos, y de forma simultánea sus efectos citotóxicos en diferentes líneas celulares (Eliades y col., 2004; David y Lobner, 2004; Vande Vannet y col., 2006, 2007; Costa y col., 2007). Mikulewicz y Chojnacka (2011b) han llevado a cabo una revisión sistemática, enfocada a la histocompatibilidad de biomateriales que contengan Ni en osteoblastos. Revisando la bibliografía científica, se recogen

a continuación algunos de los trabajos consultados, exponiendo los hallazgos más significativos

Eliades y col. (2004) caracterizaron *in vitro*, de forma cuali y cuantitativa los iones liberados a partir de series de brackets de acero inoxidable y de arcos de Ni-Ti, e investigaron de forma comparativa la citotoxicidad empleando el ensayo del MTT, y efectos sobre la síntesis del ADN sobre fibroblastos del ligamento periodontal humanos. Costa y col. (2007) investigaron los efectos citotóxicos de los extractos de corrosión de dos tipos de brackets sobre la línea celular L929 de fibroblastos de ratón, empleando el ensayo de cristal violeta para la viabilidad celular, y el ensayo del MTT para evaluar el metabolismo celular y la proliferación celular. Los resultados mostraron que ninguno de los brackets investigados alteraron la viabilidad celular, mientras que el bracket de composición tipo SS AISI 304 disminuyó la actividad metabólica mitocondrial de forma significativa a partir de los extractos obtenidos tras 42 días y de 63 días de inmersión. Estos resultados demuestran que el metabolismo celular mitocondrial puede afectarse a concentraciones bajas de Ni, mientras que se necesitan concentraciones superiores para inducir la muerte celular.

Vande Vannet y col. (2006) investigaron los efectos citotóxicos de una serie de arcos, empleando un cultivo *in vitro* multicapa de células humanas epiteliales, en lugar de un cultivo monocapa (tridimensional 3D a partir de la línea celular TR146). Ningún arco mostró citotoxicidad aguda y en concreto, los arcos de acero inoxidable indujeron menos citotoxicidad. Posteriormente, dichos autores investigaron en el mismo modelo tridimensional tres tipos de arcos de acero inoxidable soldados: soldados por un punto (PW), soldados con láser (LW) y soldados con plata (SiS), con fines de comparación. La evaluación histológica y la medida de viabilidad (MTT) mostraron que en ningún tipo de arcos se producía una severa toxicidad o pérdida de viabilidad (Vande Vannet y col. 2007).

Oh y col. (2005) investigaron la citotoxicidad de diferentes brackets de acero inoxidable mediante el método de difusión sobre agar, descrito en la norma

ISO 7405, empleando un material de cobre y polietileno como controles positivo y negativo, respectivamente. La línea celular escogida fue L-929 (fibroblastos de ratón). Tras la adición del colorante rojo neutro, e incubación de las células con los discos de la muestra, evaluaron las zonas de lisis y decoloración mediante microscopio de contraste de fases. Se compararon los resultados obtenidos para brackets de acero inoxidable, SR-50A con alto contenido de Ni, Cr y Mo, con Mini-diamond y Archist, de forma que el bracket SR-50A fue el que mostró mayor biocompatibilidad, con un índice de respuesta medio (0/1), en comparación con los otros tipos, que tuvieron índices medio-positivos.

Kao y col. (2007) evaluaron los efectos citotóxicos de cuatro brackets metálicos diferentes sobre fibroblastos humanos oral gingivales (HGF) y sobre células de un sarcoma osteogénico humano (U2OS). No se observaron microscópicamente cambios morfológicos en ambos tipos de células, y concluyeron que las células de diferente origen pueden sufrir variadas respuestas celulares tras la exposición a medios de inmersión de brackets metálicos, siendo los cuatro tipos de brackets investigados biocompatibles en ambos tipos celulares. al no evidenciarse cambios apoptóticos o de necrosis.

Además de la citotoxicidad, David y Lobner (2004) evaluaron *in vitro* los efectos neurotóxicos de diferentes arcos empleados en ortodoncia sobre células corticales de ratón. En concreto investigaron arcos de NiTi, CuNiTi., Ti-Mo, Elgiloy y de acero inoxidable, empleando como bioindicador de la muerte neuronal, la liberación de la enzima lactato deshidrogenasa tras 24 h de exposición. Los resultados indicaron que las aleaciones de NiTi, CuNiTi y TiMo no fueron neurotóxicas, mientras que el acero inoxidable y el material Elgiloy fueron significativamente tóxicos. Los autores sugieren que Fe es el elemento responsable de la toxicidad de Elgiloy, pues causa necrosis en cultivos de células corticales, mientras que Ni y/o Cr serían los responsables de la toxicidad del acero inoxidable, ya que ambos inducen apoptosis en diferentes tipos de células .

Sería muy conveniente realizar una puesta al día de los estudios y resultados realizados, sin limitaciones sobre el modelo experimental y los distintos procedimientos de exposición utilizados, conocer las deficiencias, y proponer acciones y posibilidades de investigación futuras. Indicar, que hay que tener precaución en la extrapolación de estos resultados obtenidos *in vitro*, ya que en estos ensayos se generan productos de corrosión que se añaden a las células en condiciones estáticas durante un tiempo prolongado y no mimetizan las condiciones dinámicas de un escenario *in vivo*. Por todo ello, una vez demostrada la mayor biocompatibilidad de un determinado tipo de bracket, o de aplicación ortodóncica en general, se necesitan estudios posteriores sobre la biocompatibilidad *in vivo.*

4.3. Genotoxicidad de aparatología ortodóncica y su evaluación

Las propiedades genotóxicas de los metales, a partir de las aplicaciones ortodóncicas, se definen como criterio fundamental para seleccionar estos materiales de manera segura y biológica para los pacientes (Montanaro y col., 2005). Por ello, el estudio de la genotoxicidad de las aplicaciones en ortodoncia, usualmente a base de aleaciones de acero inoxidable, que contienen metales como Cr, Ni, Co, Cu, Fe, muchos de ellos con potencial genotóxico, es un criterio fundamental a la hora de seleccionar estos materiales en relación con su biocompatibilidad, y la seguridad de los pacientes. Los estudios *in vivo* sobre la genotoxicidad de los materiales son muy escasos.

Algunos de los ensayos más empleados en la evaluación de la genotoxicidad son el ensayo del cometa, que mide roturas de las bandas simples o dobles del ADN, y el ensayo de micronúcleos (MN) que mide lesiones en los cromosomas, estando ambos considerados como biomarcadores adecuados para evaluar daños en el ADN celular. La combinación de ambos ensayos se considera muy beneficiosa, ya que aportan características suplementarias (Van Goethem y col., 1997).

· **Ensayo Cometa:**

Se trata de una prueba fundamental y determinante en la investigación de aspectos fundamentales del daño de ADN y de respuestas celulares en relación a dichas alteraciones. Se ha probado que es un indicador muy valioso en epidemiología molecular. Es un ensayo atractivo, tanto por ser rápido, fiable, sensible y económico en la medición del daño de ADN, como por sus aplicaciones, permitiendo observaciones en células simples. Actualmente, se está aplicando a la monitorización del daño de ADN en la población humana (Collins y col., 1997; Azqueta y Collins, 2013).

El ensayo cometa alcalino detecta fracturas de hebra simple o doble de ADN. El principio del ensayo cometa es que las células que poseen daño en el ADN, que han sufrido lisis tras ser incrustadas en agarosa, proporcionan el sustrato para las enzimas reparadoras o extractos celulares añadidos al gel. A diferencia de una electroforesis convencional en la que la distancia que migra el fragmento a estudiar es inversamente proporcional al tamaño de la muestra, en el ensayo cometa, el ADN no se desplaza como fragmentos. El ensayo cometa incluye la incubación del ADN a pH elevado antes y durante la electroforesis, aunque el estudio original de Ostling y Johanson (1984) empleaba pH tendente a neutro. Según Collins y col. (1997), la cantidad máxima de ADN liberado en la cola es de un 25% en el sistema neutro, mientras que bajo condiciones alcalinas puede ascender al 90%.

Muchos agentes genotóxicos no inducen las fracturas de las hebras de forma directa, sino que crean regiones álcali-lábiles y es probable que sufran la fractura del ADN mientras éste se encuentra en la solución de la electroforesis a pH elevado. Además, las fracturas están presentes de forma transitoria, cuando las células reparan las lesiones a través de una excisión de una base, o bien, de un nucleótido. Por ello, un número elevado de fracturas en el ensayo cometa indicará un daño elevado, o bien, una reparación deficiente.

Collins y col. (1997) aplicaron una modificación del ensayo cometa, utilizando enzimas específicas de la lesión, para la monitorización de la población humana en relación al daño oxidativo del ADN. Realizaron el ensayo cometa basado en el de Singh y col. (1988), que fue descrito por Gedik y col., (1992) y Collins y col., (1993), con la inclusión de la digestión con enzimas, endonucleasas del ADN, Endonucleasa III (Endo III) y Formamidopirimidina glicosilasa ((FPG), que informan del daño oxidativo en bases primidinicas y puricas, respectivamente (Collins, 2014).

El ensayo del cometa se denomina así por la forma característica cuando al ADN sale de la célula y del cuerpo celular, y permite detectar alteraciones como rotura de bandas, incompleta escisión de sitios de reparación etc., Las Figuras siguientes (Faccioni y col., 2003) muestran una comparación entre una célula normal, una célula cometa típica, y una célula en apoptosis.

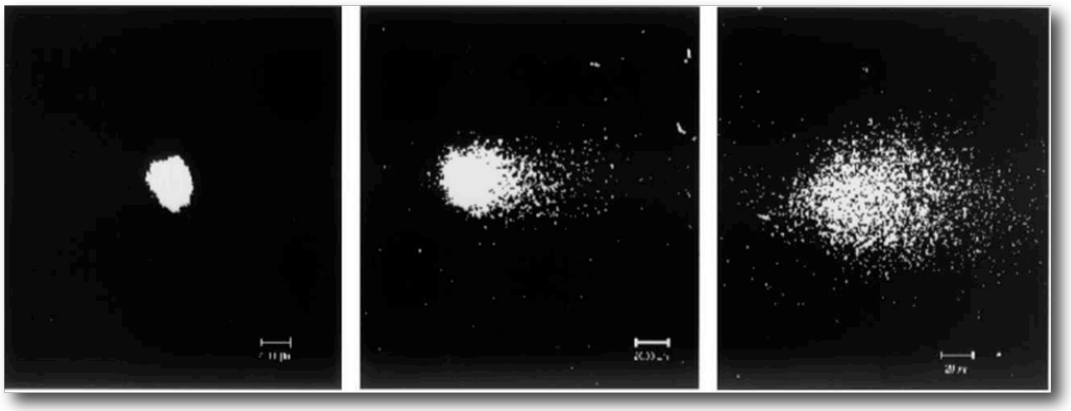

Fig. 7 **Fig. 8** **Fig. 9**

Figuras 7-9. Micrografías fluorescentes de células de la mucosa oral del ensayo cometa. Fig. 7, célula sin daño en el ADN; Fig. 8, típica célula cometa con ADN dañado; Fig. 9, célula apoptótica (Faccioni y col., 2003).

Hoy día se reconoce que existen cuatro áreas principales de investigaciones en las cuales el cometa se aplica: ensayos de genotoxicidad *in vitro* and *in vivo*, biomonitorización humana, ecogenotoxicología e investigación básica de mecanismos de daño en el ADN y mecanismos de reparación (Collins,

2004; Azqueta y col., 2011; Azqueta y Collins, 2013). Hasta donde se ha podido constatar en la literatura científica, el primer trabajo que evidenció la genotoxicidad de iones derivados de las aleaciones en ortodoncia sobre células humanas ha sido realizado por Faccioni y col. (2003). Investigaron: 1) la concentración de metales en células de la mucosa oral, 2) la biocompatibilidad, estudiando la viabilidad celular y apoptosis y 3) posible daño al ADN en las células bucales por liberación de estos metales, mediante el ensayo del cometa.

Entre las ventajas de utilizar las células bucales podemos citar: el método es el menos invasivo disponible para medir daño en el ADN *in vivo*, y estas células representarían una diana adecuada para efectos genotóxicos tempranos inducidos por agentes carcinógenos que penetran en el organismo por vía inhalatoria y por ingestión. En el estudio, se consideraron 30 individuos control y 55 pacientes con aplicaciones fijas consistentes en 4-8 bandas y 20 brackets cementados. Los materiales fueron American Iron and Steel Institute (AISI) tipo 304 para las bandas, y tipo 316 para brackets (Omrco). Los arcos fueron de aleación Ni-Ti (Ni 50,8%); Acero inoxidable (Tru-Chrome, Ni 8,6%) o aleación de Cr-Co-Ni (Elgiloy, Ni 15%, Cr 20% Co 42%, Mo 7%). Las células epiteliales de la mucosa bucal se tomaron siguiendo el método de Besarati Nia y col. (2000). Se realizó un contaje de las células, y su viabilidad se determinó empleando la técnica de exclusión de azul tripan, y dos alícuotas de las células de la mucosa bucal de cada paciente para realizar el ensayo cometa versión alcalina (Speit y Hartmann, 1999). Se evaluó el daño al ADN determinando el porcentaje de ADN en la cola (% ADN), la longitud de la cola (μm) y el momento de la cola, definido como el producto de la longitud del cometa y la cantidad de ADN en la misma. El contenido metálico se determinó mediante ICP-MS. La biocompatibilidad de los materiales se evaluó a través de las frecuencias de células en apoptosis, células con cometas y viabilidad celular. La viabilidad celular fue inferior en los pacientes con ortodoncia, existiendo correlaciones negativas significativas con los niveles de los metales. Ello indicó que las aleaciones de Ni y Co liberaron iones metálicos

en cantidades suficientes para inducir daños citotóxicos evidentes. Ambos metales indujeron daños en el ADN, existiendo correlaciones positivas entre: contenidos de Co en las células bucales y la longitud de las colas de los cometa, y entre el nº de cometas y células en apoptosis. Determinaron correlación positiva entre iones de Ni y nº de células cometa, existiendo correlación negativa entre niveles de Ni y viabilidad celular. Este estudio corroboró los potenciales efectos tóxicos de Ni y Co, que pueden producir rotura del ADN en células de la mucosa oral. Normalmente, las células pueden reparar estas lesiones, pero la pérdida de su capacidad de reparación o deficiencias enzimáticas en los procesos de reparación puede iniciar los efectos adversos.

Algunas críticas recibidas para este trabajo y los puntos que debían clarificarse estuvieron relacionadas con: 1) Las poblaciones de células no estaban caracterizadas y era posible que más de un tipo de células epiteliales con diferentes potenciales de apoptosis estuvieran agrupadas en los ensayos; 2) El análisis estadístico de los contenidos celulares de Ni y Co y la significación de las interacciones no quedó establecida.

Por todo ello se aconseja seguir con los estudios para establecer de forma inequívoca la importancia crítica de estos hallazgos. Posteriormente han sido varios los ensayos llevados a cabo, especialmente en aplicaciones ortodóncicas clásicas, como brackets etc., siendo necesario hacer una revisión actual del tema para evaluar la posible relación existente entre liberación de elementos metálicos y producción de genotoxicidad *in vivo*, y por otro lado, existe la necesidad de investigar diferentes aplicaciones ortodóncicas de uso más actual.

Ensayo de Micronúcleos (MN):

El ensayo de micronúcleos (MN) se basa en la frecuencia de micronúcleos, estructuras que se originan a partir de fragmentos de cromosoma o de cromosomas completos que no están incluidos en el núcleo principal durante la división nuclear (Fenech y col., 1999). Así, los MN surgen a partir de la fractura

de ADN, conduciendo a un cromosoma acéntrico, así como a partir de un cromosoma/cromatina que se encuentre rezagado en anafase. La formación de MN está considerada un biomarcador efectivo de enfermedades y procesos asociados con la inducción del daño de ADN.

Westpahlen y col. (2008) han investigado la genotoxicidad de algunas aplicaciones ortodóncicas fijas, empleando la combinación de los ensayos de Micronúcleos (MN) y del cometa sobre condiciones alcalinas en células bucales. El procedimiento de obtención de las muestras fue similar al de Faccioni y col. (2003), mediante cepillado de la parte interna del labio superior pero con un cepillo citológico, siguiendo las recomendaciones de Holland y col. (2008), después de lavado de la boca varias veces con agua tibia para eliminar las células muertas exfoliadas. El ensayo de MN fue llevado a cabo mediante el protocolo estandarizado de Holland y col. (2008), mediante teñido de las células y observación al microscopio. El daño primario al ADN, evaluado por el ensayo cometa fue bajo al principio, ó tras 10 días del tratamiento, no existiendo diferencias significativas en ambos periodos, demostrándose que no se induce daño genético. Sin embargo, se produjo un incremento significativo de la frecuencia de MN 30 días después de comenzar el tratamiento. La diferencia en los resultados obtenidos por ambos ensayos se basa en que miden indicadores genéticos específicos, generalmente el ensayo cometa detecta más daño en el ADN que el ensayo de MN. Los resultados positivos en el ensayo cometa no siempre se corresponden con resultados positivos en el ensayo de MN. En este estudio el ensayo de MN se mostró más sensible. Se recomienda la combinación de ambos ensayos porque: permite la detección de daño primario en el ADN (ensayo cometa), reparable, en un corto periodo de tiempo, mientras que el ensayo de MN detecta daños cromosómicos en un estadio posterior.

A partir de estos trabajos pioneros, se han sucedido diversas investigaciones *in vivo* sobre células de la mucosa oral, sobre el potencial genotóxico de las aplicaciones ortodóncicas, cuyos resultados son contradictorios.

Son estudios no estandarizados entre si, variables en el número de pacientes, con materiales diferentes, y con tiempos de medida del potencial genotóxico diverso (inicio, final tratamiento, etc.). Por ello sería muy conveniente hacer una revisión sobre el estado actual del tema, y realizar estudios experimentales *in vivo* que nos permitan avanzar en el conocimiento de esta temática.

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III. JUSTIFICACIÓN Y OBJETIVOS / SIGNIFICANT AND PURPOSES

A la vista de los antecedentes bibliográficos expuestos anteriormente, se constata la necesidad de actualizar de forma global y sistemática las últimas contribuciones al avance del conocimiento sobre la biocompatibilidad de los materiales ortodóncicos considerando las últimas aportaciones que se hayan publicado sobre la liberación de los elementos metálicos a partir de los mismos y sus principales efectos tóxicos, haciendo especial hincapié en los estudios de citotoxicidad y genotoxicidad realizados con este tipo de materiales en células de la mucosa oral, habida cuenta del potencial citotóxico y genotóxico de muchos de ellos. En este sentido, se plantearon revisiones bibliográficas que implican una puesta al día de los efectos citotóxicos y genotóxicos que pueden presentarse potencialmente tras la liberación de diversos cationes metálicos, como Ni, Cr, Co, Fe, Cu, revisando los aspectos toxicológicos más relevantes de los mismos; incluyendo una revisión sistemática enfocada sobre una de las matrices objeto de este estudio, células de mucosa oral, que incluya específicamente los efectos agudos celulares y sobre el ADN inducidos por contacto directo con las aplicaciones ortodóncicas.

Al constatarse la escasez de métodos adecuados que permitan valorar *in vivo* la liberación de elementos metálicos mediante diversas técnicas espectroscópicas, y que los aplicados hasta la actualidad carecían de validación, se decidió desarrollar, optimizar y validar métodos que permitieran cuantificar de forma simultánea, exacta y reproducible los elementos que potencialmente pueden liberarse de aplicaciones ortodoncias fijas, en células de mucosa oral de pacientes mediante la técnica de Emisión de Plasma acoplado inductivamente-Espectrometría de Masas (ICP-MS). Por otro lado, se ha considerado necesario investigar la utilidad del pelo como nueva matriz para cuantificar y valorar la exposición a metales procedentes de lixiviados a partir de aparatología ortodóncica de forma crónica, identificando así los riesgos tóxicos a largo plazo,

empleando la técnica de Espectrometría de Absorción atómica (AAS).

Debido a la implantación cada vez mayor del uso de mini-implantes en ortodoncia, nos resultó interesante identificar y valorar la posible contribución que puede suponer su empleo en la liberación de metales iónicos en la mucosa oral de pacientes, así como investigar sus posibles efectos genotóxicos *in vivo*, utilizando para ello el ensayo cometa, siendo estas investigaciones pioneras en el caso de mini-implantes.

Por todo ello, los objetivos específicos propuestos en la presente Tesis Doctoral han sido:

1. Realizar una revisión de actualización de la bibliografía científica sobre los efectos tóxicos, en particular citotóxicos, y genotóxicos inducidos por aparatología ortodóncica *in vitro*, e *in vivo*. Además, se revisará de forma sistemática la literatura científica relativa a las células de la mucosa oral, una de las matrices objeto de estudio.

2. Desarrollar y validar métodos analíticos-toxicológicos, incluyendo ensayos de robustez, que permitan la cuantificación simultánea de la liberación de elementos metálicos diversos, como Co, Cr, Cu, Ni, Ti, V y Zr a partir de aparatología ortodóncica *in vivo*, en células de mucosa oral de pacientes tratados, por Espectroscopia de Plasma inducido acoplado a Espectrometría de Masas (ICP-MS).

3. Investigar la idoneidad del pelo como matriz no invasiva que permita evaluar la potencial acumulación de metales *in vivo* en pacientes tratados con aplicaciones ortodoncias fijas, en comparación con una población control. Estudiar la posible interrelación entre dichos elementos metálicos liberados, y valorar si las posibles diferencias de los contenidos de cationes metálicos cuantificados en ambas poblaciones pueden estar

ligadas bien a factores humanos (edad, sexo), y/o factores ligados a los tratamientos (tiempo de tratamiento, maloclusión, extracción).

4. Evaluar *in vivo* la contribución que puede suponer el empleo de microtornillos en la liberación de metales en células de mucosa oral de pacientes sometidos a un tratamiento ortodóncico. Investigar posibles interrelaciones de los contenidos metálicos entre sí, y su relación con el tipo de tratamiento.
5. Poner a punto y optimizar el ensayo del cometa *in vivo* en células de mucosa oral humana y su aplicación para valorar daños genotóxicos derivados de las aplicaciones ortodoncicas fijas y mini-implantes en pacientes sometidos a dichos tratamientos.

Esta Tesis Doctoral esta estructurada como una recopilación de artículos científicos. Los tratamientos ortodóncicos y la obtención de muestras se ha llevado a cabo en las instalaciones de la Clínica Universitaria de la Facultad de Odontología de la Universidad de Sevilla. Los estudios han contado con la aprobación del Comité Ético de la Universidad de Sevilla, y con el consentimiento escrito firmado por todos los pacientes. El trabajo experimental se ha realizado en el Área de Toxicología de la Facultad de Farmacia de la Universidad de Sevilla, haciendo uso así mismo de las instalaciones de los Servicios de Biología, Microscopía y de Radioisótopos del Centro de Investigación, Tecnología e Innovación de la Universidad de Sevilla (CITIUS). Asimismo, las investigaciones sobre la determinación de metales en pelo se han llevado a cabo en los laboratorios del área de Toxicología del Departamento de Medicina Legal, Toxicología y Antropología Física de la Universidad de Granada, gracias a la colaboración de la Dra. Molina-Villalba, y muy especialmente al trabajo y

supervisión del Profesor Dr. Fernando Gil Hernández, Catedrático de Toxicología de la Universidad de Granada.

Siguiendo la normativa de la Universidad de Sevilla, el resumen, la justificación y objetivos, y las conclusiones se redactan tanto en castellano como en inglés para optar a la "Mención Internacional en el Título de Doctor".

Taking into account the scientific literature previously described, there is a need to up to date thoroughly the most recent contributions on the biocompatibility of orthodontic materials considering the last reports regarding the release of metallic elements from them and their main toxic effects, with special focus on their cytotoxicity and genotoxicity on buccal mucosa cells, as they are known to be cytotoxic and genotoxic. In this sense, different bibliographic reviews were considered: on one hand the actualization of the cytotoxic and genotoxic effects than can potentially appear after the exposure to different metallic ions, such as Ni, Cr, Co, Fe, Cu released from orthodontic appliances, reviewing their most relevant toxicological aspects and, on the other hand, a systematic review focused on one of the matrices studied, oral mucosa cells.

Once it was realized that appropriate methods to evaluate *in vivo* the release of metallic elements by different spectroscopic techniques were scarce, and that those already applied were not validated we decided to develop, optimize and validate methods that would allow to quantify in a simultaneous, exact and reproducible way, the elements that can be potentially released from fixed orthodontic appliances, in oral mucosa cells of patients by Inductively Couple Plasma-Mass Spectrometry (ICP-MS). Moreover, we considered also valuable to investigate the applicability of scalp hair as a new matrix to quantify and evaluate the exposure to metals from lixiviates of orthodontic appliances, identifying potential toxic risks after long exposures, using Atomic Absorption Spectrometry (AAS).

Due to the growing use of mini-implants in the orthodontic practice, we considered interesting to identify and evaluate the contribution of their use in the release of metallic ions in the oral mucosa cells of orthodontic patients, as well as

to investigate their potential genotoxic effects *in vivo*, using the Comet assay, an issue not previously studied.

Therefore, the specific objectives of this PhD thesis were:

1. To perform a review in order to up to date the scientific bibliography regarding to the toxic effects, particularly citotoxicity and genotoxicity, induced by orthodontic appliances both, *in vivo* an *in vitro*. Furthermore, a systematic review of the scientific literature regarding oral mucosa cells, one of the matrices studied, will be performed.

2. To develop and validate analytic-toxicological methods, including robustness assays, that allow to quantify simultaneously the release of different metallic elements such as Co, Cr, Cu, Ni, Ti, V and Zr from orthodontic appliances *in vivo*, in oral mucosa cells of orthodontic patients, by Inductively Coupled Plasma- Mass Spectrometry (ICP-MS).

3. To investigate the suitability of scalp hair as non invasive matrix to assess the potential accumulation of metals *in vivo* in orthodontic patients, in comparison to a control population. To study the potential correlation among the released metallic elements and evaluate whether the differences found in the content of metallic cations quantified in both populations could be linked to human factors (age, sex) and/or factors related to the treatment (time, malocclusion, extraction).

4. To evaluate *in vivo* the contribution of the use of mini-implants to the release of metals in oral mucosa cells of orthodontic patients. Also, to

investigate potential interrelations between metallic elements and their relation with the treatment.

5. To improve and optimize the *in vivo* Comet assay in human oral mucosa cells and their application to evaluate the genotoxic damage due to the use of fixed orthodontic appliances and mini-implants in patients with such treatments.

This PhD thesis is structured as a compendium of scientific articles. The orthodontic treatments and the sampling took place in the facilities of the University Clinic of the School of Dentistry of the University of Sevilla. The studies had the approval of the Ethical Committee of the University of Sevilla, and the written consent of all participants was obtained. The experimental work has been performed in the Area of Toxicology of the Faculty of Pharmacy (University of Sevilla) as well as in the Services of Biology, Microscopy and Radioisotopes from Centro de Investigación, Tecnología e Innovación from the University of Sevilla (CITIUS). Moreover, the studies regarding the determination of metals from hair were performed in the laboratory of the Area of Toxicology of the Department of Legal Medicine, Toxicology and Physic Anthropology of the University of Granada, thanks to the collaboration with Dr. Molina-Villalba and particularly to the efforts and supervision of Dr. Fernando Gil Hernández, full Professor of Toxicology of the University of Granada.

Following the regulations from the University of Sevilla, the summary, significance and purposes, and conclusions are written both in Spanish and in English to aim for a PhD with international mention.

IV. RESULTADOS Y DISCUSIÓN / RESULTS AND DISCUSSION

CAPÍTULO 1 / CHAPTER 1

Ana Martín-Cameán, Ángeles Jos, Ana Calleja, Fernando Gil, Alejandro Iglesias, Enrique Solano, Ana M Cameán

VALIDATION OF A METHOD TO QUANTIFY TITANIUM, VANADIUM AND ZIRCONIUM IN ORAL MUCOSA CELLS BY INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (ICP-MS).

Talanta 118, 238-244, **2011**

Talanta 118 (2014) 238–244

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Validation of a method to quantify titanium, vanadium and zirconium in oral mucosa cells by inductively coupled plasma-mass spectrometry (ICP-MS)

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ARTICLE INFO

Article history:
Received 5 July 2013
Received in revised form
7 October 2013
Accepted 15 October 2013
Available online 23 October 2013

Keywords:
Titanium
Vanadium
Zirconium
Oral mucosa cells
ICP-MS
Orthodontics appliances

ABSTRACT

The release of metal ions from fixed orthodontic appliances is a source of major concern. Various studies have evaluated the discharge of metals from these appliances in biological fluids, such as saliva or blood, overlooking the cells with prolonged contact with fixed appliances. The aim of this work is to develop and optimize an analytical procedure to determine Ti, V and Zr in oral mucosa cells in patients with and without orthodontic appliances by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The analytical procedure is based on an extraction and digestion of the samples and quantification of the elements. A suitable and practical procedure for assessing the trueness and precision of the proposed method has been applied by using validation standards. The method has been suitably validated: the regression equation was calculated from standards prepared in the same matrix without oral mucosa cells and the linear range was 0.5–50.0 ng/mL for Zr and 5.0–50.0 ng/mL for Ti and V. Limits of detection were 0.9, 2.8 and 0.4 ng/mL and limits of quantification 1.8, 3.4 and 0.7 ng/mL for Ti, V and Zr, respectively. The recovery percentages (%) obtained oscillated between 101 and 108 for Ti, 98 and 111 for V, and 92 and 104 for Zr. Intermediate precision (RSD%) data obtained were also adequate. The present method showed to be robust for the three factors considered: heating time, volume of the deionized water, and volume of PlasmaPure 65% HNO_3 used to dilute the samples, which permits its validation and application to oral mucosa cells from orthodontic patients.

1. Introduction

Orthodontic appliances biocompatibility is strongly related to ionic release. Currently there is an increasing research about the lixiviation of metal ions from biomaterials in several sites of the human body. Within the oral cavity, during an orthodontic treatment the oral cells are in full contact with metal appliances. Each orthodontic treatment lasts 24–30 months and during all this time, corrosion processes are usually present. Since the oral cavity has the proper conditions, such as humidity, pH and bacterial flora, the release of metal ions is facilitated, and that can cause adverse effects [1].

Fixed orthodontic appliances usually include brackets, bands, arch wires and springs. They are made of stainless steel, nickel–titanium or nickel–cobalt alloys [1]. Andreasen and Hilleman [2] first introduced nickel–titanium (NiTi) wires in orthodontics in the early 1970s. Such an alloy was characterized by 55% nickel and 43% titanium in terms of weight percent [3]. NiTi alloys are frequently used nowadays, especially during the levelling phase at the beginning of an orthodontic therapy with fixed appliances, because of their optimum mechanical properties [4]. Goldberg and Burstone [5] also highlighted that it is possible to make an orthodontic wire with interesting elastic properties, by processing 11% molybdenum (Mo), 6% Zr, and 4% tin beta titanium (βTi) alloys containing V. The super multifunctional titanium alloy "Gum metal" has been developed. This material, belongs to a beta-type titanium alloy having a body-centered-cubic structure and is fundamentally expressed as $Ti_3(Ta+Nb+V)+(Zr, Hf)+O$ [6]. The common criterion for all these fixed orthodontic materials is their permanent presence in the oral cavity for a long time without the ability to be removed by the own patient.

Several findings have been reported about the elemental release from many different dental casting alloys with different

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http://dx.doi.org/10.1016/j.talanta.2013.10.035

compositions. However, generalization of these statements for all dental casting alloys cannot be applied because of different reasons. First, multiple phases of the treatment will often increase the elemental release from alloys [7]. Second, certain elements have an inherently higher tendency to be released from dental alloys [7,8], and third, certain environmental conditions around the alloy will affect the elemental release [9,10].

Generally speaking for all fixed dental materials, elemental release from these materials plays a great role in their biocompatibility because they can induce adverse biological effects such as cytotoxicity, mutagenicity and allergy [11]. In this sense, various studies have evaluated the discharge of metal ions from orthodontic appliances in biological fluids, and most of them have concluded that they do not reach toxic concentrations in saliva and serum [12,13]. However, it cannot be excluded that even nontoxic concentrations might be sufficient to produce biological changes in the oral mucosa [14]. Occasionally, the host response to the elemental release differs in the nature and amount of the released elements. Moreover, classically allergic responses are characterized by dose-independence, this is, low doses that would not cause inflammation through toxicity but it would cause it by activating immune cells [15]. Also, mutagenicity and carcinogenic effects are not related with the dose of the toxicant. Therefore, knowledge about the elemental release from these materials into the oral cavity in regards to quantification is of great importance [11,16].

The release of elements from dental casting alloys has been mainly measured using either atomic absorption spectroscopy (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES), or inductively coupled plasma mass spectrometry (ICP-MS). Both techniques, ICP-AES and ICP-MS, are used for *in vivo* analysis of metals released in saliva [4,17].While ICP-AES was used with artificial oral saliva [18], ICP-MS was used with artificial oral saliva [19], cell culture medium [20], pH 3.5, pH 6 phosphate buffer solution, or pH 3.5 mixture of lactic acid and sodium chloride [21]. For many elements, the power of detection of ICP-AES is not sufficient to determine elemental background concentrations. In general, lower limits of detection (LODs) are possible to obtain by ICP-MS in comparison to ICP-AES [22]. Today, by application of ICP-MS the fast and accurate routine multi-element determination in biological samples has become possible due to improved sensitivity and robustness [22].

Compared with other biological samples, such as hair, serum, blood or urine, relatively scarce work has been done on methods for the multi-element determination in human saliva since today [4,17]. Some authors have indicated an increase in the salivary concentration of nickel (Ni) and chromium (Cr) following the insertion of fixed orthodontic appliances [12,23,24]. Saliva represents an easily accessible and useful body fluid for biomonitoring human exposure to environmental contaminants, although there is no consensus in its use for this aim [25]. Different advantages of saliva over blood collection are the following: it is non-invasive, it is the technique of choice for children and patients with limited coping abilities, its cost is lower, there is no risk of infection, and samples do not require special handling or preservation [26]. The disadvantage of saliva is related to its flow, which is influenced by many factors. Saliva flow does not influence all substance concentrations to the same degree, so it can still be a useful matrix for non-flow-dependent chemicals [25]. Moreover, saliva will give information at the moment of sampling only [27].

To the extent of our knowledge, the release of metals in oral mucosa cells, with prolonged contact with fixed appliances, has been scarcely investigated [1,27–30] and no previous validation data are available, although this matrix shows the same advantages than saliva samples previously mentioned. Moreover, no studies have been previously performed regarding Zr and V levels in this matrix. Therefore, more studies are necessary to elucidate optimal conditions to determine several metals in oral mucosa cells by ICP-MS, including robustness assays, which permit their validation, as it have been carried out in the present work. Classical approaches to analytical method validation rarely consider the stage corresponding to the robustness study, which is primary in the sense of "method transfer", according to harmonization purposes [31].

Taking all these data into account the aim of this work was to develop a rapid, sensitive and robust method, based on Inductively Coupled Plasma Mass Spectrometry (ICP-MS), suitable for simultaneously monitoring trace levels of Ti, V and Zr in oral mucosal cell samples from patients with orthodontic appliances. The procedure has been validated by using validation standards, according to González et al. [32]. Additionally, the metallic elements present in the orthodontic appliances employed were determined by micro-Xray fluorescence.

2. Materials and methods

2.1. Reagents and materials

High purity deionized water ($>$ 18 MΩ cm) obtained by a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used throughout. All transfer pipettes, centrifuge tubes, plastic bottles, autosampler vials and glassware material were cleaned by soaking in 20% v/v HNO_3 analytical reagent grade for 4 h, rinsing three times with Milli-Q water, according to EPA method 200.8 [33], and drying in a laminar flow hood.

Blank solution consisted of 1% v/v HNO_3, prepared by diluting 65% PlasmaPure nitric acid (SCP Science, Courtaboeuf, France) with the appropriate volume of Milli-Q water. A tuning solution containing 10 ng/mL cerium (Ce), cobalt (Co), lithium (Li), thallium (Tl) and yttrium (Y) in 1% HNO_3 was prepared from single-element 10000 μg/mL stock standards (AccuStandard, Inc., New Haven, CT, USA), and was used to optimize ICP-MS parameters. Rhodium 1 μg/mL, prepared from a 100 μg/mL stock solution (AccuStandard, Inc., New Haven, CT, USA) was used as internal standard solution throughout the whole analysis.

A standard solution containing 1 μg/mL of V was prepared in 100 mL Pyrex glass volumetric flask by dilution of 10 μg/mL multi-element standard solution for ICP-MS (AccuStandard, Inc., New Haven, CT, USA). Similarly, Ti and Zr standard solutions (1 μg/mL) were prepared by dilution of 1000 μg/mL single-element standard solutions (High-Purity Standards, Charlestone, SC, USA). The standard solution of 1 μg/mL was subsequently diluted to obtain working solutions (100 ng/mL or 50 ng/mL) in order to spike the digestion extracts and prepare the validation standards.

2.2. Instrumentation

All ICP-MS measurements of metal contents were carried out in an Agilent 7500c ICP-MS (Agilent Technologies, Tokio, Japan), provided with and Octupole Reaction System and an Integrated Autosampler (Agilent Technologies, Tokio, Japan). Sample introduction was performed with a Babington PEEK (poly-eter-eter-ketone) nebulizer combined with a double-pass spray chamber (Agilent Technologies, Tokio, Japan). The spray chamber was water-cooled at 2 °C to ensure temperature stability and to reduce water vapor present in the nebulizer gas flow. The ICP torch consists of a three-cylinder assembly, with injector diameter 2.5 mm. Shield torch was used throughout the whole analysis. All instrument parameters were optimized daily while aspirating the tuning solution. Typical ICP-MS operating parameters are summarized in Table 1.

These parameters were optimized to obtain the highest signal-to-background ratio for ^{7}Li, ^{59}Co, ^{89}Y, ^{140}Ce and ^{205}Tl, as well as

minimizing the oxides ($^{140}Ce^{16}O^+/^{140}Ce^+$), hydrides ($^{140}CeH^+/^{140}Ce^+$) and doubly-charged ($^{140}Ce^{++}/^{140}Ce^+$) signals.

Micro-X-ray fluorescence (μXRF) measurements were performed in an EAGLE III [energy-dispersive analysis by X-rays (EDAX)] energydispersive micro-X-ray fluorescence spectrometer equipped with a Rh X-ray tube, 300-μm monocapillary optics, a charge-coupled device (CCD) camera, and an 80-mm^2 Si (Li) detector. Surface scans of 0.5 cm^2 were performed under a vacuum with a data acquisition time of 150 s. The quantification limit was 0.1%, and the elements that could be measured were those between Na and Pu. The apparatus was previously calibrated according to the manufacturer's specification using an aluminum–copper standard sample. Automated analyses were performed by using the fundamental parameter quantification routine.

2.3. Sample collection and sample preparation

Forty subjects were included in this study. Twenty patients required fixed orthodontic treatment (orthodontic group or test group), and 20 subjects served as the control group who were not undergoing orthodontic treatment. Both groups were similar regarding the sex of the components: 10 men and 10 women in the control group, and 13 women, 7 men in the orthodontic group. The ages range was 17–46 years in the control group and 12–53 in the orthodontic group. The time for orthodontic treatment of patients was 13–15 months. The orthodontic patients were all treated with fixed orthodontic appliances in both arches. The appliances consisted of 8 bands on the first and second molars, 20 brackets and 12 patients used 0.016×0.022 nickel–titanium archwires in upper and lower arches, whereas 8 patients used 0.016×0.022 stainless steel archwires in both arches. The archwires were fixed with 0.010 stainless steel ligatures and all the patients had long 0.012 stainless steel ligatures. The aims and the method of cell collection were explained to all subjects, and written consent to participate was obtained. Treatment was started after the institutional ethical committee of the University of Seville approved the protocol. The fixed of appliances consisted of an average of 4–8 bands or tubes and 20 bonded brackets. The material used was stainless steel alloy SAF2205, AISI316L and AISI303 for the brackets, tubes and bands (DM Ceosa; Madrid, Spain). The ligatures were made of stainless steel alloy AISI304. The archwires used in this study were nickel–titanium alloy (DM Ceosa, Madrid, Spain) or stainless steel (DM Ceosa, Madrid, Spain). The materials used in this study were analyzed by μXRF, and their compositions are shown in Table 2.

The inclusion criteria for subject selection included non smokers; no oral diseases, no systemic diseases, no oral restorations or prosthetic; clinically healthy oral mucosa; no previous orthodontic treatment; no occupational exposure to metals, and not receiving any medication or supplements. Subjects were initially screened with a questionnaire to check whether they fit the criteria of the study. Afterwards they were clinically assessed for normal oral mucosa [27].

The participants were asked to rinse their mouth with tepid water for 1 min to remove exfoliated dead cells. Epithelial cells of buccal mucosa from each patient were collected, using a mini-toothbrush (Difresh, Madrid, Spain), according to the method of Besaratinia et al. [34], by gentle brushing of the internal part of the oral mucosa in contact with the orthodontic appliances.

Once the samples were collected, they were digested and measured following the method of Natarajan et al. [29] with some modifications regarding the water and nitric acid volumes employed, the heating time (60 min instead of 30 min), and the use of an internal standard. Briefly, toothbrush was introduced into a previously cleaned (4 h in 20% v/v HNO_3) 50 mL centrifuge tube, together with 10 mL of deionized water and 100 μL of PlasmaPure 65% HNO_3. Then, samples were heated in a water bath at 80 °C for 60 min. Afterwards, samples were cooled lightly and sonicated in an ultrasonic bath for 5 min. Finally, samples were cooled down to room temperature, and the acid solution was separated from the brush. Acid solutions were stored in clean 20-mL polypropylene vials at 4 °C until analysis. 5 mL of the sample volume was required for the analysis. The amounts of Ti, V and Zr in the cells were quantitatively assessed by ICP-MS. The addition of internal standard (^{103}Rh), was performed on line.

Extraction efficiencies were performed in triplicate by spiking the matrix, clean toothbrush without oral mucosal cells submitted to the same extraction procedure, with the multi-element standard solution at three concentration levels: 1, 10 and 50 ng/mL for Zr, and 5, 10 and 50 ng/mL for Ti and V. Besides, a robustness study was carried out by spiking the matrix with a standard solution of 25 ng/mL of each analyte.

Table 1
ICP-MS instrument parameters.

Parameter	Setting
RF Power (W)	1500
RF Matching (V)	1.80
Sampling depth (mm)	4.6
Carrier gas (L/min)	1.15
Spray chamber temperature (°C)	2
Nebulizer pump (revolutions per second, rps)	0.1
Extract (V)	3.8
Einzel 1,3 (V)	−100
Einzel 2 (V):	22
Cell entrance (V)	−50
Cell exit (V):	−47
Plate bias (V)	−44
QP bias (V)	−4.5
OctP RF (V)	190
OctP bias (V)	−7.0

Table 2
Chemical composition of the orthodontic appliances used in the study.

Material – Product	Composition (wt%)
Stainless steel – Ligature.010	18.93 Cr, 0.50 Cu, 70.37 Fe, 0.39 Mo, 9.58 Ni, and 0.23 Rb
Stainless steel – Ligature.012	18.77 Cr, 0.30 Cu, 70.57 Fe, 0.21 Mo, 9.94 Ni, and 0.20 Rb
Band single tube	17.51 Cr, 0.60 Cu, 69.59 Fe, 2.06 Mo, 9.75 Ni, 0.29 Rb, and 0.19 V
Band double tube	18.66 Cr, 0.31 Cu, 68.80 Fe, 2.22 Mo, 9.63 Ni, 0.19 Rb, and 0.18 V
Bracket BioMesh	18.42 Cr, 0.37 Cu, 66.94 Fe, 2.47 Mo, 11.57 Ni, and 0.23 Rb
Tube	18.18 Cr, 0.50 Cu, 67.95 Fe, 2.30 Mo, 10.83 Ni, and 0.23 Rb
TMA arch	18.33 Cr, 0.53 Cu, 72.71 Fe, 0.27 Mo, 7.99 Ni, and 0.18 Rb
0.014 Nickel–titanium arch	0.03 Cr, 56.36 Ni, 43.49 Ti, and 0.12 Zr
0.016 Nickel–titanium arch	0.12 Cr, 56.33 Ni, 43.42 Ti, and 0.13 Zr
0.016x.022 Stainless steel arch (AISI302 alloy)	18.74 Cr, 0.61 Cu, 72.38 Fe, 0.24 Mo, 7.41 Ni, and 0.62 Co

2.4. Statistical criteria calculations for method validation

The study of intermediate precision and trueness was performed by applying an one-factor ANOVA (GraphPad InStat software Inc., La Jolla, USA) between days. Three validation standards covering the optimal working range (0.5–50 ng/mL) were used. Each validation standard was measured in quintuplicate for two different days. From the ANOVA results, as explained in "Section 3", both the intermediate precision and the recovery were obtained. The values have been compared with tabulated reference values.

The robustness study was carried out using an intermediate validation standard (25 ng/mL of each metal) according to the Youden procedure [35]. The influential factors (the heating time employed, the volume of the deionised water used to dilute the samples, and volume of PlasmaPure 65% HNO_3 employed) were tested according to the Student *t*-test as indicated below.

Data of metal content from oral mucosal cells of patients (control and orthodontic patients) are expressed as mean ± standard deviation. Data distribution was always found non-normal, and accordingly, non-parametric methods were applied. Dunn test was used for comparing the individual treatments. Statistical significance was inferred at $P < 0.05$ (GraphPad InStat software Inc., La Jolla, USA).

3. Results and discussion

3.1. General aspects

In order to develop the ICP-MS method for the detection of Ti, V and Zr in oral mucosa cells, commercially available calibration standards solutions of the three elements were prepared by diluting the appropriate volume of a 10 μg/mL mixed-element working standard with blank solution, to a final concentrations of 0.5, 1, 5, 10, 50, 100 and 250 ng/mL of each element. These calibration standards were used to assess the linear calibration range of the instrument,. It was found that, at least between 0.5 and 250 ng/mL range, the response of the ICP-MS was linear. The concentration of the internal standard was 300 ng/mL Rh in all sample and calibration standard solutions.

Selected isotopes were ^{47}Ti, ^{51}V and ^{90}Zr. Three-points-per-mass peak pattern was chosen, and measurements were carried out in three replicates. Integration times per point, and per mass, were 0.2 s and 0.6 s, respectively, for the three elements. Integration time for internal standard was 0.01 s per point and 0.03 per mass.

3.2. Method validation

3.2.1. Linear range

The response as a function of concentration of each metal was measured by at least 5-point calibration curve with a range within 0.5–50 ng/mL. In all cases, the ratios CPS analyte/CPS internal standard (being CPS counts per second) were recorded as signal. Response linearity was established according to Huber [36] by plotting the called response factors (signal response/analyte concentration) against their respective concentrations. Responses were obtained from five or six mini-toothbrush introduced in clean 50 mL centrifuge tubes and suitably spiked with working standards of Ti, V and Zr, and submitted to the digestion and ICP-MS proposed procedure in triplicate. The Huber plots obtained for Ti, V and Zr are shown in Fig. 1. The target line has zero slope and the intercept is just the median of the response factors obtained. Two parallel horizontal lines are drawn in the graph at 0.95 and 1.05 times the median value of the response factors in a fashion similar to the action limits of control charts. As no intersections with the lines were found in the case of zirconium, the linear range of the method applies to the full range studied, 0.5–50.0 ng/mL. In the case of Ti and V, the adequate linear range found for both elements was 5.0–50.0 ng/mL. These values are similar to those found by Natarajan et al. [29] when they analyzed nickel and chromium concentrations on oral mucosa cells in a range of 1–40 ng/mL of both elements, using ICP-MS. Fernández-Miñano et al. [30] evaluated in vivo metal ions release from three alloys, but they did not provide any validation data.

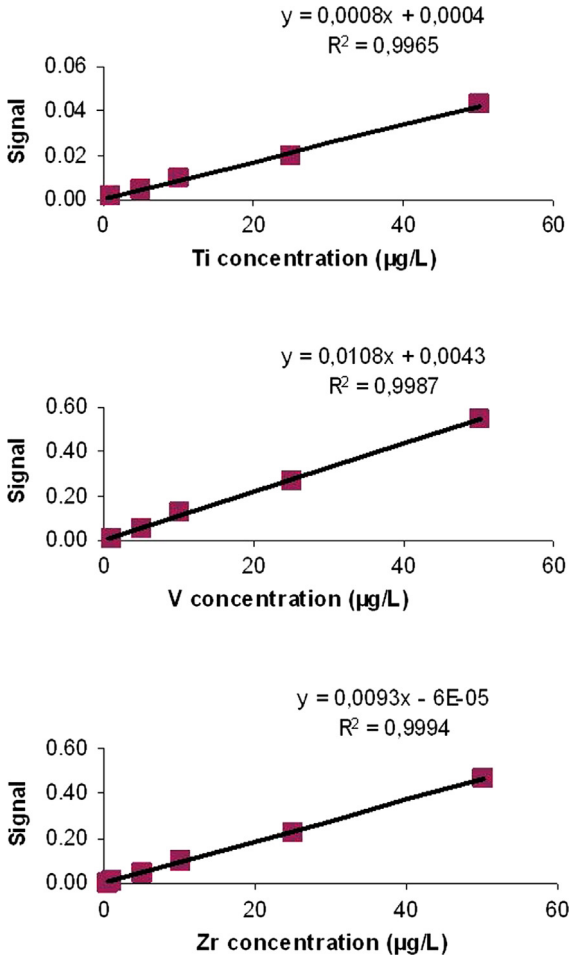

Fig. 1. Linear calibration functions for the proposed procedure.

3.2.2. Goodness of the fit

The linear calibration function was obtained by preparing five or six calibration standards in the digestion extracts resulting from the mini-toothbrush introduced in clean 50 mL centrifuge tubes (in triplicate) from 0.5 to 50 ng/mL of Ti, V and Zr, and recording the signal response according to the proposed digestion and ICP-MS procedure. Here, mini-toothbrush treated with 10 mL of deionized water and 100 μL of PlasmaPure 65% HNO_3 are taken as blank samples and the analytes (Ti, V and Zr) are spiked in order to obtain similar conditions for future samples. So, these calibration standards can be also considered as validation standards (VS). The calibration lines have correlation coefficients of 0.9965, 0.9987, and 0.9994 for Ti, V, and Zr, respectively (Fig. 2), and there is not lack-of fit and the calibration functions can be considered as linear.

3.2.3. Detection and quantitation limits

The limit of detection (LOD) and the LOQ were determined, by measuring 10 independent sample blanks. Limit of detection was

estimated using the expression $Y_{LOD}=Y_{blank}+3S_{blank}$, where Y_{blank} and S_{blank} are the average value of the blank signal and its corresponding standard deviation. Limit of detection values are then converted into concentration by using the calibration function. The procedure for evaluating LOQ was equivalent to that of LOD, but using the factor 10 instead of three for calculations. The LOD obtained were 0.9, 2.8, and 0.4 ng/mL for Ti, V and Zr, respectively. The LOQ for three elements assayed were 1.8, 3.4 and 0.7 ng/mL for Ti, V and Zr, respectively. To the extent of our best knowledge, no data of these parameters have been previously reported in the determination of trace metals in oral mucosa cells using this technique (ICP-MS). Similar detection limits (1 ng/mL) were found by Amini et al. [1] which analyzed nickel, chromium and cobalt in oral mucosa cells using atomic absorption spectrometry with graphite furnace (AAS-GF). As far as we know, no LOD and LOQ data were available for the three elements considered in the scientific literature.

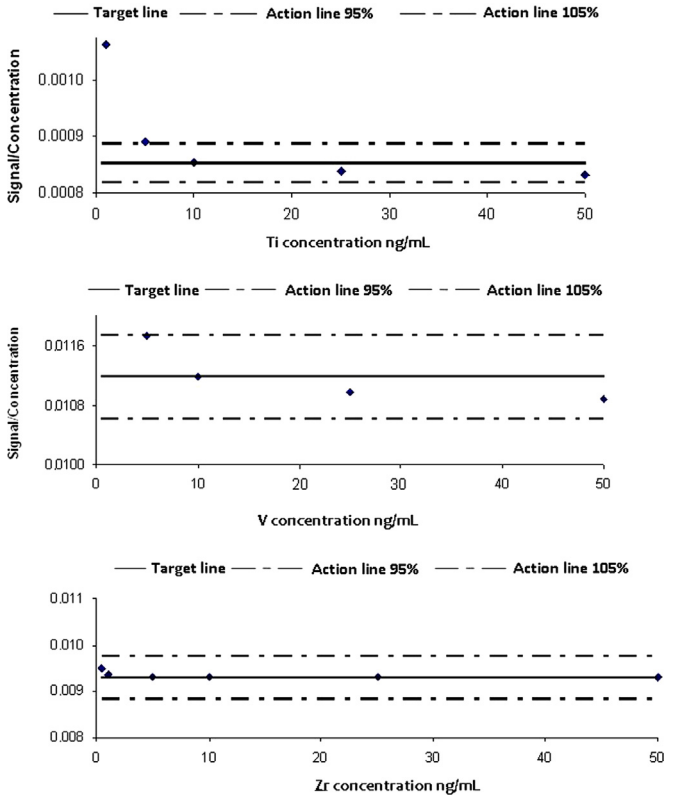

Fig. 2. Huber plots for assessing linear range.

3.3. Accuracy study

3.3.1. Intermediate precision and trueness studies

According to the International Conference on Harmonization guidelines [37], precision may be considered at three levels: repeatability, intermediate precision, and reproducibility. Repeatability expresses the precision evaluated under the same experimental conditions over a short time interval, and it is termed as intra-assay or within-run. Intermediate precision applies to within-laboratory variations: different days, different analysts or equipments and it is sometimes called between-run or inter-assay precision [32].

On the other hand, the trueness of an analytical procedure expresses the closeness of agreement between the mean value obtained from a series of measurements and the value, which is accepted either a conventional value or an accepted reference value like validation standards [32].

Repeatability and intermediate precision were calculated analyzing five replicates of mini-toothbrush spiked at three validation standards of the three metals considered (low, medium and high) covering the dynamic working range (0.5–50.0 ng/mL) on the same day and in two different days, respectively.

Considering two different days, as the main source of variation, an analysis of variance (ANOVA) was performed for each concentration, obtaining estimations of within-condition variance (S_w^2), also known as repeatability (S_r^2), and between-condition variance (S_B^2). Also, the intra laboratory reproducibility or intermediate precision, is obtained as $S_{IP}^2=S_r^2+S_B^2$ [31,32]. All these parameters are shown in Table 3.

From these data, the corresponding relative standard deviations, RSD_R were calculated and compared with the acceptable RSD percentages obtained from the AOAC Peer Verified Methods (PVM) program [32,36]. As a quick rule [32], the RSD_{IP} results should be compared with one-half the corresponding RSD values tabulated. Our results for Ti, V and Zr, at the three concentration levels considered, were lower or the same order than the one-half $\%RSD_{AOAC}$ tabulated for each element (Table 3).

The assessment of trueness can be performed according the same ANOVA results. Trueness can be expressed as the bias or recovery obtained for each validation standards assayed [38]. The recovery term has a more intuitive meaning and it has been tested in this work. The total recovery for any validation standards is defined as the ratio between the observed estimation of the validation standards concentration, and the "true" value T, expressed as percentage or as fraction. The recoveries (%) computed for the three validation standards considered for each element are shown in Table 3. We checked them for suitability by comparison with the published acceptable recovery ranges as a function of the analyte concentration [32,36]. In our method, as the Ti and Zr concentrations of the three validation standards ranged between 1 and 50 ng/mL,

Table 3
Estimations of within-condition (repeatability), between-condition, intermediate precision (intra laboratory reproducibility) and recoveries of titanium, vanadium and zirconium assayed at three validation standards, in two different days.

Paramenters	Ti concentration level			V concentration level			Zr concentration level		
	5 ng/mL	10 ng/mL	50 ng/mL	5 ng/mL	10 ng/mL	50 ng/mL	1 ng/mL	10 ng/mL	50 ng/mL
S_w	0.14	0.47	1.74	0.37	0.77	1.20	0.11	0.85	1.18
S_B	0.29	0.06	4.87	0.41	0.24	2.26	0.27	0.82	2.89
S_{IP}	0.20	0.39	3.15	0.38	0.64	1.63	0.18	0.84	1.93
RSD_{IP} (%)	10.00	3.55	6.20	6.90	5.80	3.30	15.00	8.10	3.90
½ RSD_{AOAC} (%)[a]	15	11	7.5–11	11–15	11	7.5–11	15	11	7.5–11
Recovery (%)	101 ± 9	108 ± 1	102 ± 3	111 ± 3	111 ± 1	98 ± 1.5	92 ± 8	104 ± 2	99 ± 2
	Between (40–120)[b]	Between (60–115)[b]	Between (60–110)[b]	Between (40–115)[b]	Between (60–115)[b]	Between (60–110)[b]	Between (40–120)[b]	Between (60–115)[b]	Between (60–110)[b]

[a] RSD values obtained from the AOAC Peer Verified Methods program according to the concentration level of analyte ([32]).
[b] Acceptable recovery percentages according to the concentration level of analyte ([32]).

the recovery ranges (%) could oscillate between 40 and 120%, 60 and 115%, and 60 and 110%, for 1 ng/mL, 10 ng/mL and 50 ng/mL, respectively. The recoveries obtained oscillated between 101 and 108% for Ti, and between 92 and 104% for Zr. In the case of V the recoveries oscillated between 98 and 111%. All the recovery data fulfill the rule previously mentioned, and the method can be considered bias-free.

In summary, this procedure has been successfully assessed for trueness, intermediate precision and repeatability.

3.3.2. Robustness study

Robustness, considered in the sense of internal validation, deals with the effect of experimental variables, called factors, inherent in the analytical procedure (e.g., temperature, digestion conditions, pH, etc.) on the analytical result. A robustness study examines the alteration of these factors, as expected in a transfer between laboratories, so it is of the utmost importance in the uncertainty budget. The strategy for carrying out our robustness study is based on a landmark procedure suggested by Youden [35], according to the practical guide of González and Herrador [31]. Three influential factors in the sample preparation procedure were identified: (X_1) heating time employed; (X_2) volume of the deionized water used to dilute the samples, and (X_3) volume of PlasmaPure 65% HNO_3 employed. The levels are coded according to the rule: high value $=+1$ ($X_1=70$ min; $X_2=10.1$ mL; $X_3=200$ μL), and low level $=-1$ ($X_1=60$ min; $X_2=10.0$ mL; $X_3=100$ μL). The effect of every factor is estimated as the difference of the mean result obtained at the level $+1$ from that obtained at the level -1. Once effects have been estimated, to determine whether variations have a significant effect on the results, a significance t-test is used [39], and the t-values (X_k) are compared with the 95% confidence level two-tailed tabulated value with the degrees of freedom coming from the precision study for each concentration. In the present study, the experiments were carried out using validation standards spiked with 25 ng/mL of each metal considered (Ti, V and Zr), and each factor was analyzed by quintuplicate in two different days. So, for 9 degrees of freedom, the t-values obtained for X_1, X_2 and X_3 factors are shown in Table 4. In all cases, $t(X_k) < t_{tab}$ (2.262), and therefore the procedure can be considered as robust against the three factors considered (at the levels fixed in the study) for Ti, V and Zr determination.

3.4. Evaluation of titanium, vanadium and zirconium in patients with and without fixed orthodontic appliances

The cellular contents of the three elements from 40 patients, 20 of the control group and 20 of the orthodontic group, according to the proposed and validated method, were measured. The median values obtained for titanium concentration were 3.80 and 2.50 ng/g in orthodontic and control groups, respectively. Moreover, the mean value in control group (5.14 ± 3.90 ng/g) was similar to that found in orthodontic patients (5.23 ± 3.50 ng/g) and no significant differences were detected. Patients using NiTi arches showed slightly increased Ti values in comparison to patients wearing stainless steel arches, but no significant differences were found. Only traces of Zirconium were detected in the orthodontic group (0.54 ± 0.30 ng/g) and control group (0.32, lesser than the detection limit), and no significant differences were found between them. Vanadium was not detected in either the orthodontic group or the control group. These results are consistent with the minor presence of this metal in the composition of the orthodontic materials employed in this study (0.18–0.19% only in the case of bands), but the method would be suitable for monitoring emergent materials, such as Gum metal [6].

In comparison to other *in vivo* studies, our results are in agreement with the previous study by Natarajan et al. [29], in which the presence of Ni and Cr ions in the experimental group were not significantly higher than those in the control group. By contrast, Faccioni et al. [28] reported 3.4-fold and 2.8-fold increases in Ni and Co concentrations in oral mucosa cells of orthodontic patients. Fernández-Miñano et al. [30] reported that buccal cells that had been in contact with stainless steel showed higher concentrations of Ti^{47} and Mn^{55} than the control cells. Amini et al. [1], only found Ni contents significantly higher in mucosa cells of orthodontic patients compared with their non-appliance controls, and they did not report differences in chromium (Cr) and cobalt (Co) cell contents. Hafez et al. [27] reported that fixed orthodontic appliances for 6 months increased the Ni and Cr contents of the buccal mucosa cells. All these findings indicated that to ensure the safety of patients, further research would be needed to determine the long-term significance of metals release. Consequently, the development and validation of methods which permit their quantification in oral mucosa cells, which seemed advantageous because they are in direct contact with the appliances, is of great interest.

Table 4
Significance t-values (X_k) obtained in the robustness study assayed for the three elements.

Elements	X_1	X_2	X_3
Ti	0.737	0.169	0.614
V	0.101	0.157	0.095
Zr	0.667	1.428	1.985

Critical t-value = 2.262
X_1: heating time employed
X_2: volume of the deionized water used to dilute the samples
X_3: volume of PlasmaPure 65% HNO_3

4. Conclusions

In summary, we have developed and validated a method for titanium, vanadium and zirconium determination in oral mucosa cells from orthodontic patients in comparison to control patients, using a digestion procedure and quantification by ICP-MS. The procedure has been successfully assessed for trueness and precision, and can be considered as robust against the three factors considered in the digestion procedure, such as the heating time employed, the volume of the deionized water used to dilute the samples and volume of PlasmaPure 65% HNO_3 employed. The proposed method could be suitable for monitoring of these metals in buccal mucosa cells of orthodontic patients, as routine method to test the biocompatibility of fixed orthodontic appliances, and for *in vivo* studies focused in the discharge of metals from this kind of appliances.

Acknowledgment

The authors wish to thank the Junta de Andalucía (PAIDI CTS-358) for the financial support for the present study. We also thank the X-ray laboratory of CITIUS (University of Seville) for the microfluorescence analysis.

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CAPÍTULO 2 / CHAPTER 2

Ana Martín-Cameán, Ángeles Jos, Ana Calleja, Fernando Gil, Alejandro Iglesias-Linares, Enrique Solano, Ana M Cameán

DEVELOPMENT AND VALIDATION OF AN INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (ICP-MS) METHOD FOR THE DETERMINATION OF COBALT, CHROMIUM, COPPER AND NICKEL IN ORAL MUCOSA CELLS.

Microchemical Journal 114, : 73-79, **2011**

Microchemical Journal 114 (2014) 73–79

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Development and validation of an inductively coupled plasma mass spectrometry (ICP-MS) method for the determination of cobalt, chromium, copper and nickel in oral mucosa cells

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ARTICLE INFO

Article history:
Received 10 November 2013
Accepted 9 December 2013
Available online 17 December 2013

Keywords:
Metals
Oral mucosa cells
ICP-MS
Orthodontic appliances

ABSTRACT

The oral cavity is an ideal environment for the corrosion of fixed orthodontic appliances, leading to the release of metal ions that, eventually, could derive in adverse effects. Therefore, it is necessary to evaluate the biocompatibility of these materials for patient's safety. In vivo, oral mucosa cells are a valuable sample for this aim, however, analytical methods to quantify the liberation of metal ions are very scarce. Thus, the purpose of this work is to optimize and validate a sample preparation procedure to determine cobalt (Co), chromium (Cr), copper (Cu), and nickel (Ni) in oral mucosa cells in patients with and without orthodontic appliances, based on the extraction and digestion of the samples and quantification of the elements by inductively coupled plasma mass spectrometry (ICP-MS). The method has been suitably validated: the regression equation was calculated from standards prepared in the same matrix without oral mucosa cells and the linear range was 2.0–100.0 ng mL^{-1} for all elements. Limits of detection were 0.10, 0.38, 0.49 and 0.67 ng mL^{-1} and limits of quantification were 0.20, 1.13, 0.98, and 1.81 ng mL^{-1} for Co, Cr, Cu, and Ni, respectively. The recovery percentages (%) obtained ranged between 104 and 109 for Co, 103–107 for Cr, 106–113 for Cu and 84–110 for Ni. Intermediate precision (RSD%) data obtained were also adequate. The present method proved to be robust for the three factors considered: heating time, volume of deionized water, and volume of PlasmaPure 65% HNO_3 used to dilute the samples. Thus, the proposed method passed in a satisfactory way the validation standards considered and could be used to evaluate in vivo the metal ion release in oral mucosa cells from orthodontic patients.

1. Introduction

Orthodontic appliances are commonly used in dentistry to correct teeth and jaws that are positioned improperly. These intraoral fixed orthodontic appliances include brackets, bands, and archwires that are made of alloys containing nickel (Ni), cobalt (Co), and chromium (Cr) in different percentages, and also iron and copper. The different types of orthodontic archwires contain 15% to 54% Ni, 20% to 30% Cr and 40% to 60% Co [1,2].

It is well known that all metals and alloys are subject to corrosion, being electrochemical breakdown the most common type. In this context, orthodontic alloys emit electrogalvanic currents with saliva as the medium, leading to a release of metal ions on the mouth's mucosa [3]. Therefore, resistance to corrosion in the mouth is a fundamental aspect of biocompatibility. Several factors might affect, such as the manufacturing process, type of alloy, surface characteristics of the piece, environment in which the piece is inserted, and use of the alloy (aging) [4]. Moreover, the oral environment is particularly ideal for biodegradation of metals because of its ionic, thermal, microbiologic, and enzymatic properties [5,6].

One of the roles of Ni in the alloys is to increase the strength, ductility, and resistance to general, crevice and erosion corrosion. Cr ions provide an electrochemically formed passive film that offers protection against aggressive ions in the oral environment and also prevents corrosion. However, it has been reported that the major corrosion products are Fe, Cr, and Ni for stainless steel, and Ti and Ni for nickel–titanium alloys [7].

The general belief that there is no frank concern regarding the corrosion by-products released in orthodontic patients is not actually supported. Metals are not biodegradable, and their sustained leakage might produce irreversible toxic effects from their accumulation in the tissues [8]. Moreover, it has been reported that metal ions are taken up by the adjacent oral tissues [1,7,9].

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0026-265X/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.microc.2013.12.009

Nickel toxic effects are well known although it is considered an essential trace element. It is one of the most common causes of allergic contact dermatitis [10,11]. Ni compounds are classified as human carcinogens (group 1) by the International Agency for Research on Cancer [12]. Chromium and Co ions can also cause hypersensivity and dermatitis. These metals can induce cytotoxicity and genotoxicity [12–14]. Copper has been reported to be more cytotoxic than Ni [15]. Also, some heavy metals used in the fabrication of archwires, such as Fe, Cu, and Co undergo redox cycling, therefore directly producing free radicals, whereas Ni produces free radicals indirectly [16]. Actually, orthodontic archwires containing Cu induce oxidative stress in vitro, although copper–nickel–titanium and rhodium–coated nickel–titanium showed relatively lower toxicity compared with conventional nickel–titanium [2].

Various studies have evaluated the release of metal ions from orthodontic appliances in biologic fluids, and most have concluded that they do not reach toxic concentrations. However, it cannot be excluded that even nontoxic concentrations might be sufficient to produce biologic changes in the oral mucosa [17].

Taking all these into account the monitoring of the release of metal ions from orthodontic appliances is of interest. In this regard, there are different available techniques including atomic absorption spectroscopy (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES), or inductively coupled plasma mass spectrometry (ICP-MS). Nowadays, by application of ICP-MS the fast and accurate routine multi-element determination in biological samples and other matrices has become possible due to improved sensitivity and robustness [18,19].

Hair, serum, blood and urine are the conventional biological samples used for metal content determination. However, other samples such as saliva or mucosa cells have been reported to be useful to determine the metal release from orthodontic appliances. Thus, different authors have indicated an increase in the salivary concentration of Ni [20] and Cr following the insertion of fixed orthodontic appliances [6,21–23]. However, the disadvantage of saliva in comparison to oral mucosa cells is related to its flow, which is influenced by many factors. Saliva flow does not influence all substance concentrations to the same degree, so it can still be a useful matrix for non-flow-dependent chemicals [24]. Moreover, saliva will provide information at the moment of sampling only [8].

Regarding metal content determination in oral mucosa cells, reports are also very scarce [1,7,8,17,25] and results are contradictory. It is important to highlight that, to the extent of our knowledge, no previous validation data are available on this matrix, including robustness assays, apart from the study of our research group on the validation of an ICP-MS method to determine titanium, vanadium and zirconium in oral mucosa cells from orthodontic patients [26].

The aim of the present study was to optimize and validate a rapid, sensitive and robust sample preparation procedure to determine simultaneously Co, Cr, Cu, and Ni in oral mucosa cells in patients with and without orthodontic appliances, based on the extraction and digestion of the samples and quantification of the elements by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). A suitable and practical procedure for assessing the trueness and precision of the proposed method has been applied by using validation standards, according to González et al. [27]. The present procedure has been intended for evaluating the in vivo potential release of these metals to oral mucosa cells from fixed orthodontic appliances.

2. Materials and methods

2.1. Reagents and materials

High purity deionized water (>18 MΩ·cm) obtained by a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used throughout. All transfer pipettes, centrifuge tubes, plastic bottles, autosampler vials and glassware material were cleaned by soaking in 20% v/v HNO_3 analytical reagent grade for 4 h, rinsing three times with Milli-Q water, according to EPA method 200.8 (United States Environment Protection Agency, version 5.4, 1994), and drying in a laminar flow hood.

Blank solution consisted of 1% v/v HNO_3, prepared by diluting 65% PlasmaPure nitric acid (SCP Science, Courtaboeuf, France) with the appropriate volume of Milli-Q water. A tuning solution containing 10 ng mL^{-1} cerium (Ce), cobalt (Co), lithium (Li), thallium (Tl) and yttrium (Y) in 1% HNO_3 was prepared from single-element 10,000 μg mL^{-1} stock standards (AccuStandard, Inc., New Haven, CT, USA), and was used to optimize ICP-MS parameters before each analytical run. Rhodium 1 μg mL^{-1}, prepared from a 100 μg mL^{-1} stock solution (AccuStandard, Inc., New Haven, CT, USA) was used as an internal standard solution throughout the whole analysis.

A standard solution containing 1 μg mL^{-1} of Co, Cr, Cu and Ni was prepared in 100 mL Pyrex glass volumetric flask by dilution of 10 μg mL multi-element standard solution for ICP-MS (AccuStandard, Inc., New Haven, CT, USA) with the suitable amount of blank solution. The standard solution of 1 μg mL^{-1} was subsequently diluted with the suitable amount of blank solution to obtain working solutions (100 ng mL^{-1} or 50 ng mL^{-1}) in order to spike the digestion extracts and prepare the validation standards of 1, 2, 3, 5, 10, 25, 50, and 100 ng mL^{-1}.

2.2. Instrumentation

All ICP-MS measurements of metal contents were carried out in an Agilent 7500c ICP-MS (Agilent Technologies, Japan), provided with and Octupole Reaction System and an Integrated Autosampler (Agilent Technologies, Japan). Sample introduction was performed with a Babington PEEK (poly-eter-eter-ketone) nebulizer combined with a double-pass spray chamber (Agilent Technologies, Japan). The spray chamber was water-cooled at 2 °C to ensure temperature stability and to reduce water vapor present in the nebulizer gas flow. The ICP torch consists of a three-cylinder assembly, with injector diameter 2.5 mm. Shield torch was used throughout the whole analysis. All instrument parameters were optimized daily while aspirating the tuning solution. Typical ICP-MS operating parameters are summarized in Table 1.

These parameters were optimized to obtain the highest signal-to-background ratio for ^{7}Li, ^{59}Co, ^{89}Y, ^{140}Ce and ^{205}Tl, as well as minimizing the oxides ($^{140}Ce^{16}O^{+}/^{140}Ce^{+}$), hydrides ($^{140}CeH^{+}/^{140}Ce^{+}$) and doubly-charged ($^{140}Ce^{++}/^{140}Ce^{+}$) signals. The $^{140}Ce^{16}O^{+}/^{140}Ce^{+}$ signal was minimized to 0.8%.

Table 1
Typical ICP-MS instrument parameters.

Parameter	Setting
RF[a] power (W)	1500
RF matching (V)	1.80
Sampling depth (mm)	4.6
Carrier gas (L min^{-1})	1.15
Spray chamber temperature (°C)	2
Nebulizer pump (revolutions per second, rps)	0.1
Extract (V)	3.8
Einzel 1,3 (V)	−100
Einzel 2 (V):	22
Cell entrance (V)	−50
Cell exit (V)	−47
Plate bias (V)	−44
QP[b] bias (V)	−4.5
OctP[c] RF (V)	190
OctP bias (V)	−7.0

[a] RF: Radiofrequency.
[b] QP: Quadrupole.
[c] OctP: Octupole.

2.3. Sample collection and preparation

Forty subjects were included in this study. Twenty patients required fixed orthodontic treatment (orthodontic group or test group), and 20 subjects served as the control group who were not undergoing orthodontic treatment. The inclusion criteria for subject selection in both groups included non-smokers; no oral diseases, no systemic diseases, no oral restorations or prosthetic; clinically healthy oral mucosa; no previous orthodontic treatment; no occupational exposure to metals, and not receiving any medication or supplements.

Subjects were initially screened with a questionnaire to check whether they fit the criteria of the study. Afterwards they were clinically assessed for normal oral mucosa [8]. The duration of the orthodontic treatment of patients was 13–15 months. The aims and the method of cell collection were explained to all subjects, and written consent to participate was obtained. Treatment was started after the institutional ethical committee of the University of Seville approved the protocol. The fixed of appliances consisted of an average of 4–8 bands or tubes and 20 bonded brackets. The material used was stainless steel alloys SAF2205, AISI316L and AISI303 for the brackets, tubes and bands, (DM Ceosa; Madrid, Spain). The ligatures were made of stainless steel alloy AISI304. The archwires used in this study were nickel–titanium alloys (DM Ceosa, Madrid, Spain) or stainless steel (DM Ceosa, Madrid, Spain). The materials used in this study were analyzed by Micro-Xray fluorescence (μXRF) [26]. The source of Cr and Ni comes from all the materials employed in the orthodontic treatment: stainless steel ligatures, bands, brackets, tubes, and arches (TMA, nickel–titanium and stainless steel). Similarly, Cu proceeds from the orthodontic appliances with the exception of nickel–titanium arches. Finally, the potential release of Co is from stainless steel arches.

The participants were asked to rinse their mouth with tepid water for 1 min to remove exfoliated dead cells. Epithelial cells of buccal mucosa from each patient were collected, using a soft rubber interdental brush without any metal content, as stated by the manufacturer (Sunstar Iberia S.L., San Just Desvern, Spain), and following the method of Besarati Nia et al. [28], by gentle brushing of the internal part of the oral mucosa in contact with the orthodontic appliances. After reviewing the scientific literature several sampling instruments were found to be of common use, such as wooden tongue depressors [8], metal spatula [17] or interdental brushes [7,28]. Preliminary analysis was carried out in our laboratory in order to assess the suitability of the different sampling tools for the intended determinations. Noticeable metal background was found in the case of mini toothbrush and interdental brush with metallic parts (data not shown).

Once the samples were collected, the amount of cell suspension containing approximately 1000 cells was calculated and then they were digested and measured following the method of Natarajan et al. [17] with some modifications regarding the water and nitric acid volumes employed, the heating time (60 min instead of 30 min), and the use of an internal standard. Briefly, each interdental brush was introduced into a previously cleaned (4 h in 20% v/v HNO_3) 50 mL centrifuge tube, together with 10 mL of deionized water and 100 μL of PlasmaPure 65% HNO_3. Then, samples were heated in a water bath at 80 °C for 60 min. Afterwards, samples were cooled lightly and sonicated in an ultrasonic bath for 5 min. Finally, samples were cooled down to room temperature, and the acid solution was separated from the brush. Acid solutions were stored in clean 20-mL polypropylene vials at 4 °C until analysis. 5 mL of the sample volume was required for the analysis. The amounts of metals assayed in the cells were quantitatively assessed by ICP-MS. The addition of the internal standard (^{103}Rh) was performed on-line. The same procedure was applied to the clean interdental brushes without sample in order to obtain the methodological blank.

Extraction efficiencies were performed in triplicate by spiking the matrix, clean interdental brush without oral mucosa cell submitted to the same extraction procedure, with the multi-element standard solution at three concentration levels: 5, 10 and 50 ng mL^{-1} for each element. Besides, a robustness study was carried out by spiking the matrix with a standard solution of 25 ng mL^{-1} of each analyte.

2.4. Statistical criteria calculations for method validation

The study of intermediate precision and trueness was performed by applying a one-factor ANOVA (GraphPad InStat software Inc., La Jolla, USA) between days. Three validation standards covering the optimal working range (1.0–100.0 ng mL^{-1}) were used. Each validation standard was measured in quintuplicate in two different days. From the ANOVA results, as explained in the "Results and discussion" section, both the intermediate precision and the recovery were obtained. The values have been compared with tabulated reference values.

The robustness study was carried out using an intermediate validation standard (25 ng mL^{-1} of each metal) according to the Youden procedure [29]. The influential factors (the heating time employed, the volume of the deionized water used to dilute the samples, and the volume of PlasmaPure 65% HNO_3 employed) were tested according to the Student *t*-test as indicated below.

Data of metal content from oral mucosa cell of patients (control and orthodontic patients) are expressed as mean ± standard deviation, and median and range of values obtained were also reported. Data distribution was always found non-normal, and accordingly, non-parametric methods were applied. Dunn test was used for comparing the individual treatments. Statistical significance was inferred at $p < 0.05$ (GraphPad InStat software Inc., La Jolla, USA).

3. Results and discussion

3.1. General aspects

In order to develop the ICP-MS method for the detection of Co, Cr, Cu and Ni in oral mucosa cells, commercially available calibration standards solutions of the four elements were prepared by diluting the appropriate volume of a 10 μg mL^{-1} mixed-element working standard with blank solution, to final concentrations of 1, 2, 3, 5, 10, 25, 50 and 100 ng mL^{-1} of each element. These calibration standards were used to assess the linear calibration range of the instrument. It was found that, at least between 1.0 and 100 ng mL^{-1} range, the response of the ICP-MS was linear. The internal standard Rh solution (1 μg mL^{-1}), was added online to every blank, validation standard and sample.

Selected isotopes were ^{59}Co, ^{53}Cr, ^{65}Cu and ^{60}Ni. Three-points-per-mass peak pattern was chosen, and measurements were carried out in three replicates. Integration times per point, and per mass, were 0.2 s and 0.6 s, respectively, for the four elements. Integration time for internal standard was 0.01 s per point and 0.03 per mass.

3.2. Method validation

3.2.1. Linear range

The response as a function of concentration of each metal was measured by at least 5-point calibration curve with a dynamic range of two orders of magnitude (1.0–100.0 ng mL^{-1}). In all cases, the ratios CPS analyte/CPS internal standard (being CPS counts per second) were recorded as signal. Response linearity was established according to Huber [30] by plotting the called response factors (signal response/analyte concentration) against their respective concentrations. Responses were obtained from eight spiked interdental brush with working standards of Co, Cr, Cu and Ni, introduced in clean 50 mL centrifuge tubes and submitted to the digestion and ICP-MS proposed procedure in triplicate. The Huber plots obtained for the metals assayed are shown in Fig. 1. The target line has zero slope and the intercept is just the median of the response factors obtained. Two parallel horizontal lines are drawn in the graph at 0.95 and 1.05 times the median value of the response factors in a fashion similar to the action limits of control charts. For all elements, the adequate linear range of the method found was 2.0–

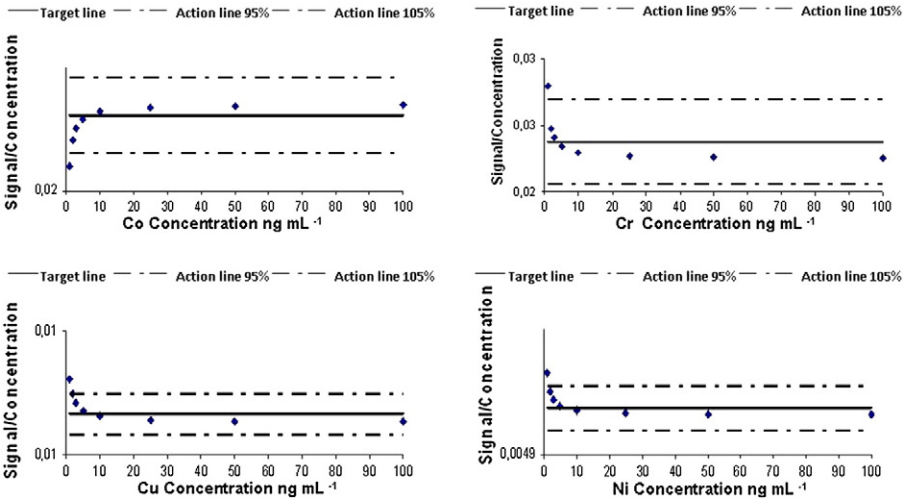

Fig. 1. Huber plots for assessing linear range.

100.0 ng mL^{-1}. These values are similar to those found by Natarajan et al. [17] when they analyzed Ni and Cr concentrations on oral mucosa cells in a range of 1 to 40 ng mL^{-1} of both elements, using ICP-MS.

3.2.2. Goodness of the fit

Taking into account the previous section, the linear calibration function was obtained by preparing seven calibration standards in the digestion extracts resulting from the interdental brush introduced in clean 50 mL centrifuge tubes (in triplicate) from 2.0 to 100 ng mL^{-1} of each element assayed, and recording the signal response according to the proposed digestion and ICP-MS procedure. Here, interdental brush treated with 10 mL of deionized water and 100 μL of PlasmaPure 65% HNO_3 are taken as blank samples and the analytes (Co, Cr, Cu and Ni) are spiked in order to obtain similar conditions for future samples. So, these calibration standards can be also considered as validation standards (VS). The calibration lines have correlation coefficients of 0.9964, 0.9996, 0.9997 and 0.9997 for Co, Cr, Cu and Ni, respectively (Fig. 2). The corresponding ANOVA of the regression lines indicates a lack-of fit F ratio of 14.11, 1.56, 1.05, and 0.12, against a critical F value of 19.4 (Fig. 2). Consequently, there is not lack-of fit and the calibration functions can be considered as linear.

3.2.3. Detection and quantitation limits

The limit of detection (LOD) and the limit of quantification (LOQ) were determined, by measuring 10 independent interdental brush blanks. Limit of detection was estimated using the expression $Y_{LOD} = Y_{blank} + 3S_{blank,}$ where Y_{blank} and S_{blank} are the average value of the blank signal and its corresponding standard deviation. Limit of detection values were then converted into concentration by using the calibration function. The procedure for evaluating LOQ was equivalent to that of LOD, but using the factor 10 instead of 3 for calculations. The LODs obtained for the four elements were 0.10 ng mL^{-1} for Co, 0.38 ng mL^{-1} for Cr, 0.49 ng mL^{-1} for Cu, and 0.67 ng mL^{-1} for Ni. Similarly, the LOQs were 0.20, 1.13, 0.98, and 1.81 ng mL^{-1} for Co, Cr, Cu and Ni, respectively. To the extent of our best knowledge, no data of these parameters have been previously reported in the determination of trace metals in oral mucosa cells using this technique (ICP-MS). Similar and higher detection limits (1 ng mL^{-1}) were found by Amini et al. [7] which analyzed Ni, Cr and Co in oral mucosa cells using atomic absorption spectrometry with graphite furnace (AAS-GF).

3.3. Accuracy study

3.3.1. Intermediate precision and trueness studies

According to the International Conference on Harmonisation guidelines [31], precision may be considered at three levels: repeatability, intermediate precision, and reproducibility. Repeatability expresses the precision evaluated under the same experimental conditions over a short time interval, and it is termed intra-assay or within-run. Intermediate precision applies to within-laboratory variations: different days, different analysts or equipments and is sometimes called between-run or inter-assay precision [27].

On the other hand, the trueness of an analytical procedure expresses the closeness of agreement between the mean value obtained from a series of measurements and the value, which is accepted either a conventional value or an accepted reference value like validation standards [27].

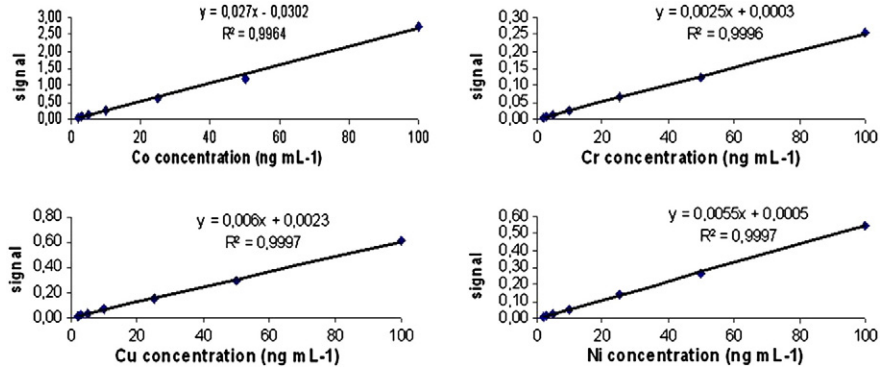

Fig. 2. Linear calibration functions for the proposed procedure for the metals assayed.

Repeatability and intermediate precision were calculated analyzing five replicates of interdental brush spiked at three validation standards of the four metals considered (low, medium and high) covering the dynamic working range (2.0–100.0 ng mL^{-1}) on the same day and in two different days, respectively.

Considering two different days, as the main source of variation, an analysis of variance (ANOVA) was performed for each concentration, obtaining estimations of within-condition variance (S^2_w), also known as repeatability (S^2_r), and between-condition variance (S^2_B). Also, the intra-laboratory reproducibility or intermediate precision, can be estimated as the sum $S^2_{IP} = S^2_r + S^2_B$ [27,32]. All these parameters are shown in Table 2.

From these data, the corresponding relative standard deviations, RSD_R, were calculated and compared with the acceptable RSD percentages obtained from the AOAC Peer Verified Methods (PVM) program [27,30]. As a quick rule [27], the RSD_{IP} results should be compared with one-half the corresponding RSD values tabulated. Our results for Co, Cr, Cu and Ni, at the concentration levels considered, were lower or the same order than the one-half $\%RSD_{AOAC}$ tabulated for each element (Table 2).

The assessment of trueness can be performed according the same ANOVA results. Trueness can be expressed as the bias or recovery obtained for each validation standard assayed [33]. The recovery term has a more intuitive meaning and it has been tested in this work. The total recovery for any validation standard is defined as the ratio between the observed estimation of the validation standards concentration, and the "true" value T, expressed as percentage or as fraction. The recoveries (%) computed for the three validation standards considered for each element are shown in Table 2. We checked them for suitability by comparison with the published acceptable recovery ranges as a function of the analyte concentration by the AOAC Peer Verified Methods program [27,30]. In our case, the recoveries obtained oscillated between 104 and 109% for Co, 103–107% for Cr, 106–113% for Cr, and 84–110% for Ni. All the recovery data fulfill the rule previously mentioned, and the method can be considered bias-free.

In summary, this procedure has been successfully assessed for trueness, intermediate precision and repeatability.

3.3.2. Robustness study

Robustness, considered in the sense of internal validation, deals with the effect of experimental variables, called factors, inherent in the analytical procedure (e.g., temperature, digestion conditions, pH, etc.) on the analytical result. A robustness study examines the alteration of these factors, as expected in a transfer between laboratories, so it is of the utmost importance in the uncertainty budget. The strategy for carrying out our robustness study is based on a landmark procedure suggested by Youden [29], according to the practical guide of González and Herrador [32]. Three influential factors in the sample preparation procedure were identified: (X_1) heating time employed; (X_2) volume of the deionized water used to dilute the samples, and (X_3) volume of PlasmaPure 65% HNO_3 employed. The levels are coded according to the rule: high value = +1 (X_1 = 70 min; X_2 = 10.1 mL; X_3 = 200 μL), and low level = −1 (X_1 = 60 min; X_2 = 10.0 mL; X_3 = 100 μL). The effect of every factor is estimated as the difference of the mean result obtained at the level +1 from that obtained at the level −1. Once effects have been estimated, to determine whether variations have a significant effect on the results, a significance *t*-test is used [34], and the t-values (X_k) are compared with the 95% confidence level two-tailed tabulated value with the degrees of freedom coming from the precision study for each concentration. In the present study, the experiments were carried out using validation standards spiked with 25 ng mL^{-1} of each metal considered (Co, Cr, Cu, and Ni), and each factor was analyzed by quintuplicate in two different days. So, for 9° of freedom, the t-values obtained for X_1, X_2 and X_3 factors are shown in Table 3. In all cases, $t(X_k) < t_{tab}$ (2.262), and therefore the procedure can be considered as robust against the three factors considered (at the levels fixed in the study) for determination of the metals considered.

Table 3
Significance t-values (X_k) obtained in the robustness study for the elements assayed.

	X_1	X_2	X_3
Co	0.468	0.218	0.093
Cr	1.571	0.388	0.360
Cu	0.153	0.085	0.017
Ni	1.760	1.407	0.822

Critical t-value = 2. 262.
X_1: heating time employed.
X_2: volume of the deionized water used to dilute the samples.
X_3: volume of PlasmaPure 65% HNO_3.

3.4. Evaluation of cobalt, chromium, copper and nickel in patients with and without fixed orthodontic appliances

The measured cellular contents of Co, Cr, Cu and Ni from 40 patients, 20 of the control group and 20 of the orthodontic group, according to the proposed and validated method, are shown in Table 4. In all cases, the mean ion metal values showed significant increases in the orthodontic group in comparison with the control group. The mean values of metal concentrations in the oral mucosa cell of orthodontic patients were increased approximately 13.5-fold, 9.5-fold, 2.0 fold, and 6.3-fold for Co, Cr, Cu and Ni, respectively. Although previous studies have evaluated metal ions in the saliva, few studies have estimated the ion content in oral mucosa cell quantitatively. In comparison to these scarce in vivo studies, our results are in agreement with the results reported by Faccioni et al. [1], which found 3.4-fold and 2.8-fold increases in Ni and Co concentrations in oral mucosa cells of orthodontic patients after 2–4 months of treatment. By contrast, Natarajan et al. [17] studied the release of Ni and Cr ions from fixed orthodontic appliances (n = 20 patients) at two times: at debonding and 30 days after debonding, and the Ni and Cr concentrations were not significantly different from normal patients, although the Cr ion concentration was higher in the experimental group at 30 days after debonding. Amini et al. [7], found Ni

Table 2
Estimations of within-condition (repeatability), between-condition, intermediate precision (intra laboratory reproducibility) and recoveries of cobalt, chromium, copper and nickel assayed at three validation standards, in two different days.

	Co (ng mL^{-1})			Cr (ng mL^{-1})			Cu (ng mL^{-1})			Ni (ng mL^{-1})		
	5	10	50	5	10	50	5	10	50	5	10	50
S_w	0.34	0.36	1.32	0.28	0.37	1.14	0.32	0.66	0.99	0.51	0.43	0.81
S_B	0.02	0.68	3.78	0.10	0.72	3.11	0.25	0.36	3.91	0.06	0.51	2.14
S_{IP}	0.28	0.49	2.43	0.24	0.52	2.02	0.30	0.58	2.40	0.75	0.46	1.40
RSD_{IP} (%)	5.10	4.50	4.70	4.40	4.70	3.90	5.20	5.20	4.50	7.50	4.10	2.70
1/2 RSD_{AOAC} (%)[a]	11–15	11	7.5–11	11–15	11	7.5–11	11–15	11	7.5–11	11–15	11	7.5–11
Recovery (%)	108 ± 1 (40–120)[b]	109 ± 1 (60–115)[b]	104 ± 1 (60–115)[b]	107 ± 1 (40–120)[b]	107 ± 1 (60–115)[b]	103 ± 1 (60–115)[b]	113 ± 0.1 (40–120)[b]	111 ± 1 (60–115)[b]	106 ± 1 (60–115)[b]	84 ± 1 (40–120)[b]	110 ± 1 (60–115)[b]	103 ± 1 (60–115)[b]

[a] RSD values obtained from the AOAC Peer Verified Methods program according to the concentration level of analyte [27].
[b] Acceptable recovery percentages according to the concentration level of analyte [27].

Table 4
Concentrations of metal ions (ng mL^{-1}) detected in oral mucosa cells in control and orthodontic patients.

	Control group (n = 20)			Orthodontic group (n = 20)			
Metal	Mean ± SD	Median	Range	Mean ± SD	Median	Range	p values
Co	0.9 ± 1.1	0.6	ND–2.5	12.7 ± 6.3	11.6	4.1–25.8	0.0003***
Cr	2.9 ± 3.1	2.3	ND–7.2	28.2 ± 20.1	17.5	10.9–65.0	0.0035**
Cu	5.1 ± 1.0	4.9	4.3–6.4	11.0 ± 7.2	8.5	5.5–30.0	0.0304*
Ni	4.6 ± 2.6	4.3	1.9–7.9	29.1 ± 15.4	24.9	10.6–63.0	0.0008***

* Significant at $p < 0.05$.
** Significant at $p < 0.01$.
*** Significant at $p < 0.001$.

contents significantly higher in the mucosa cells of orthodontic patients compared with their non-appliance controls, but they did not report differences in Co and Cr cell contents. Fernández-Miñano et al. [25] only detected more Cr and Fe in mucosa cells of patients in the nickel-free group (n = 5) in comparison to control group (n = 15). Hafez et al. [8] reported that fixed orthodontic appliances for 6 months increased the Ni and Cr contents of the buccal mucosa cells. Reports on the Cu release from orthodontic appliances in oral mucosa cells have been not found in the scientific literature. However, Staffolani et al. [35] observed in vitro that Cu was released from orthodontic appliances dipped in both inorganic and organic acid solutions. In our study, none of the orthodontics patients presented clinical symptoms, and the increases in metal concentrations were lower when compared with their respective Tolerable daily intake (TDI) in the case of Co (1.4 μg kg^{-1} body weight [36], Cu (5 mg day^{-1}) [37] and Ni (12 μg kg^{-1} body weight) [38], or with the Tolerable Upper Intake level (UL) established for Cr (1 mg day^{-1}) by the Scientific Committee of Food of the European Commission [37]. All these results indicated that to ensure the safety of patients, further research would be needed to determine the long-term significance of metal release. Consequently, the development and validation of methods which permit their quantification in oral mucosa cells, an advantageous sample as they are in direct contact with the appliances, are of great interest.

4. Conclusions

An ICP-MS method with a previous digestion procedure has been optimized and validated for the simultaneous determination and quantification of Co, Cr, Cu and Ni in oral mucosa cells from orthodontic patients in comparison to control patients. The procedure has been successfully assessed for trueness and precision, and can be considered as robust against the three factors considered in the digestion process. This method would allow to acquire more information about the in vivo corrosive potential of intraoral appliances.

Acknowledgment

The authors wish to thank the Junta de Andalucía (PAIDI CTS-358) for the financial support of the present study.

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CAPÍTULO 3 / CHAPTER 3

Ana Martín-Cameán, Isabel Molina-Villalba, Ángeles Jos, Alejandro Iglesias-Linares, Enrique Solano, Ana M Cameán, Fernando Gil

BIOMONITORIZATION OF CHROMIUM, COPPER, IRON,MANGANESE AND NICKEL IN SCALP HAIR FROMORTHODONTIC PATIENTS BY ATOMIC ABSORPTION SPECTROMETRY.

Environmental Toxicology and Pharmacology 37, :759-777, **2014**

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/etap

Biomonitorization of chromium, copper, iron, manganese and nickel in scalp hair from orthodontic patients by atomic absorption spectrometry

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ARTICLE INFO

Article history:
Received 7 January 2014
Accepted 31 January 2014
Available online 13 February 2014

Keywords:
Metals
Scalp hair
Orthodontic appliances
Exposure assessment
Biomonitoring

ABSTRACT

The study was aimed to assess Cu, Cr, Fe, Mn and Ni levels in human scalp hair from a broad population group treated with orthodontic appliances ($n = 70$) to determine, whether the concentration of a given metal was significantly influenced by the orthodontic treatment in comparison to control group ($n = 56$). Levels of metal compounds were determined by atomic absorption spectrometry. The mean, ranges, median and 5th and 95th percentiles of metals analyzed in hair that were hypothesized to be systemically absorbed from stainless steel, are provided. The influence of individual factors on metal concentrations was considered (gender, age), and inter-element interactions were studied by evaluation of correlation coefficients between elements, as well as by multiple regression analysis. Differences in the content of metals in hair were only significantly increased for Mn when compared to the control group, but their levels were of the same magnitude to other control populations, and consequently, no risks linked to the treatment have been found. The orthodontic treatment increased significantly Mn levels in young patients (<20 years old) when compared with control group. Scalp hair analysis is a good method to investigate the release of the elements from fixed orthodontic appliances.

1. Introduction

There is an increasing concern about the biocompatibility of dental materials and, therefore, this topic has been widely investigated during recent years. The metals that are released from biomaterials in various sites of the human body have attracted the interest of many investigators because it is believed that the degradation products can elicit a foreign-body reaction or induce pathologic processes (Macedo and Cardoso, 2010). The oral environment is particularly ideal for biodegradation of metals because of its ionic, thermal,

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http://dx.doi.org/10.1016/j.etap.2014.01.025

microbiologic and enzymatic properties (Barrett et al., 1993). In this context, orthodontic alloys emit electrogalvanic currents with saliva as the medium, leading to a release of metal ions on the mouth' mucosa (Hwang et al., 2001).

The intraoral fixed orthodontic appliances include brackets, bands, and archwires that are made of alloys containing nickel (Ni), chromium (Cr) in different percentages, and also manganese (Mn), iron (Fe) and copper (Cu) (Iijimaa et al., 2006; Regis et al., 2011). The different types of orthodontic archwires contain 15–54% Ni, 20–30% Cr and 40–60% Co (Faccioni et al., 2003; Spalj et al., 2012). Several factors might affect metal release, such as the manufacturing process, type of alloy, surface characteristics of the piece, environment in which the piece is inserted and use of the alloy (aging) (Macedo and Cardoso, 2010).

Metals are not biodegradable, and their sustained leakage might produce irreversible toxic effects from their accumulation in the tissues (Hafez et al., 2011). Various studies have evaluated the release of metal ions from orthodontic appliances in biologic fluids, and most have concluded that they do not reach toxic concentrations. However, it cannot be excluded that even nontoxic concentrations might be sufficient to produce biologic changes in the oral mucosa (Natarajan et al., 2011). In fact, Ni and Cr can cause hypersensitivity, dermatitis, asthma and cytotoxicity (Goyer and Clarkson, 2001; Eliades et al., 2004; Noble et al., 2008; Pazzini et al., 2009; Arenholt-Bindsley et al., 2009). In addition, they have a significant carcinogenic and mutagenic potential (de Souza and de Menezes, 2008; IARC, 1990) and at nontoxic concentrations are able to induce DNA alterations (Faccioni et al., 2003). All these effects are not related with the dose of exposure. Elements such as Cu, Fe and Mn are usually accepted as essentials, but for their risk assessment, it is require to consider both toxicity from excess exposures and health consequences as a result of deficiencies (Goyer and Clarkson, 2001). Thus, at the cellular level, Fe overload in adults increased lipid peroxidation, with consequent membrane damage to mitochondria, microsomes and other cellular organelles. There are few reported cases of Mn toxicity from oral ingestion, but chronic exposure produces neuropsychiatric disorders. Taking all these into account, the monitoring of the release of metal ions from orthodontic appliances is of interest.

Besides serum, blood and urine are used as conventional biological samples for metal content determination (Gil and Pla, 2001; Gil and Hernández, 2009). Saliva and hair are other non-invasive matrices employed in human biomonitoring (Esteban and Castaño, 2009; Olmedo et al., 2010; Gil et al., 2011). Investigators have indicated an increase in the salivary concentration of Ni (Petumenou et al., 2009) and Cr following the insertion of fixed orthodontic appliances (Kocadereli et al., 2000; Eliades et al., 2003; Fors and Persson, 2006; Sahoo et al., 2011). Mucosa cells have been also reported to be useful to determine the metal release from orthodontic appliances (Faccioni et al., 2003; Amini et al., 2008; Natarajan et al., 2011; Hafez et al., 2011; Fernández-Miñano et al., 2011; Martin-Cameán et al., 2014). Human hair has proved to be a vehicle of excretion of substances from the human body, including heavy metals whose concentrations are up to 10-fold higher than the levels found in blood or urine (Bader et al., 1999; Olmedo et al., 2010). The high affinity of hair to metals is mainly due to the presence of cystine, which makes up approximately 14% of human hair, and metallic cations form bonds with the sulphur of the keratin matrix of the hair. According to the Environmental Protection Agency (EPA), human hair is one of the most important biological materials for worldwide environmental monitoring (Morton et al., 2002).

Human hair is a stable matrix that presents numerous advantages for human biomonitoring, such as easy collection, low cost, easy transport and storage, does not show storage changes for the period between sampling and analysis, and provides information about short- and long-term exposure (Barbosa et al., 2005; Angerer et al., 2007; Zhang et al., 2007; Gil et al., 2011), and the temporal exposure pattern by segmental analysis (Esteban and Castaño, 2009). The main disadvantages of this matrix are the difficulty in differentiating between external and internal exposure, and the lack of sufficient information to define a normal range of metal levels typically found in the general population because hair metal content vary significantly according to age, sex, hair color, and racial/ethnic factors (Barbosa et al., 2005; Angerer et al., 2007; Esteban and Castaño, 2009; Gil et al., 2011).

In general, analytical procedures for the determination of metals in hair are very scarce (Petersen et al., 2000), and recently, a validated method for the quantification of several heavy metals (Cd, Cr, Mn, Ni and Pb) in human hair has been reported by our research group by atomic absorption spectrometry (AAS) (Olmedo et al., 2010), and applied in occupationally exposed population with biomonitoring purposes (Gil et al., 2011). It is important to highlight that, to the extent of our knowledge, only a study has investigated the exposure of patients to metal released from orthodontic appliances *in situ*, using Inductively coupled plasma (ICP-OES) (Mikulewicz et al., 2011). These authors concluded that differences in the content of metals in hair between exposed and non-exposed groups were not statistically significant, because it was a preliminary work in which a small number of participants were included.

Taking all these into account, the aim of the present study was to investigate the levels of exposure to metals (Cu, Cr, Fe, Mn and Ni) in human hair sampled from a broad population group treated with orthodontic appliances to determine, whether the concentration of a given metal was significantly influenced by the orthodontic treatment in comparison to control group. Also, the influence of individual factors on metal concentrations was considered (gender, age), and inter-element interactions were studied by evaluation of correlation coefficients between elements, as well as by multiple regression analysis.

2. Materials and methods

2.1. *Population study*

A total number of 126 subjects were selected to participate in the study. 70 patients (46 females and 24 males) required fixed orthodontic treatment (orthodontic group or test group) and 56 subjects (32 females and 24 males) served as the control group who were not undergoing orthodontic treatment. The time for orthodontic treatment of patients was at least 24 consecutive months. Characteristics of the sampling method used

to collect the hair samples for this study have been previously described (Olmedo et al., 2010). Written consent was obtained from the subjects who participated in this study and they were also informed of their right to withdraw anytime, after which they all participated voluntarily. The proposal was previously approved by the Ethics Review Committee of the University of Sevilla. The fixed of appliances consisted of an average of 4–8 bands or tubes and 20 bonded brackets. The material used was stainless steel alloy SAF2205, AISI316L and AISI303 for the brackets, tubes and bands (DM Ceosa; Madrid, Spain). The ligatures were made of stainless steel alloy AISI304. The archwires used in this study were nickel–titanium alloy (DM Ceosa, Madrid, Spain) or stainless steel (DM Ceosa, Madrid, Spain). Subjects were initially screened with a questionnaire to check whether they fit the criteria of the study (non smokers, no oral and systemic diseases and not receiving any medication or supplements, no oral restorations or prosthetic, no previous orthodontic treatment and non occupational exposure to metals).

2.2. Hair samples

Regarding human hair samples, approximately 0.1 g was cut from the back of the head as close as possible to the scalp. The length varied between 1 and 4 cm. The hair samples were kept in acid precleaned polyethylene containers and the hair was washed by ultrasonic cleaning in a non-ionic detergent (Triton X-100, Merck, Darmstadt, Germany) solution, and then the detergent was removed by copious rinse with Milli-Q water and washed by ultrasonic cleaning in an ethanol solution (Merck, Darmstadt, Germany), and again with Milli-Q water. The cleaned hair was dried at room temperature overnight. After addition of 1 mL of HNO_3 (Merck), 0.5 mL of HCl (Merck), 2 mL of H_2O_2 (Merck) and 2 mL of H_2O were digested during 30 min in a microwave oven Multiwave 3000 (Anton Parr, Graz, Austria) (for more details, see Olmedo et al., 2010).

2.3. Determination of metals

The validation of analytical procedures developed for the determination of metal compounds in human hair samples has been reported elsewhere by our team (Olmedo et al., 2010). This validation protocol included the limit of detection (LOD) and quantification (LOQ), linear range, precision (minimal, intermediate and reproducibility), accuracy, recovery and characteristic mass. The analytical method was controlled by using external certified reference materials NIES N° 5 for hair obtained from the National Institute for Environmental Studies, Japan Environment Agency (Olmedo et al., 2010).

Briefly, levels of metal compounds were determined by a Perkin-Elmer Analyst 800 Atomic Absorption Spectrometer (Perkin Elmer, Norwalk, USA) equipped with flame atomization (for Cu and Fe), Zeeman background correction and an AS-800 autosampler by graphite furnace (for Cr, Mn and Ni) and graphite tubes with integrated L'vov platform (Perkin Elmer). Appropriate matrix modifiers (see Olmedo et al., 2010) were used for the selected heavy metal studied and prepared in 0.2% (v/v) nitric acid and 0.1% Triton X-100. Prior dilution of each sample was critical in order to obtain the best results. The limit of detection (LOD) was <0.001 mg/g for Cu and 0.021, 6.640, 0.013 and 0.026 μg/g for Cr, Fe, Mn and Ni, respectively. Calibration graphs were linear until 5 mg/L for Cu and Fe, and 30, 20 and 15 μg/L for Cr, Mn and Ni, respectively.

2.4. Statistical analysis

Statistical analysis of the results was performed by the software Statistica (version 6.0, Statsoft, Inc., OK, USA). Descriptive statistics (means, standard deviation, medians, range and 5–95th percentiles) were reported. Normality of distribution of experimental results was assessed by Chi-Square test. Data distribution was always found non-normal, and accordingly, non-parametric methods were applied. Thus, differences in mean levels of metal compounds as well as the potential influence of classical confounders (gender and age) on these levels were assessed by using the Mann–Whitney test. The magnitude of the correlation between metal concentration in human hair and these factors was carried out by determination the non-parametric technique of Spearman. Also, a multiple regression analysis was performed including potential confounding clinical variables that might influence the concentration of metals. Therefore a stepwise linear regression model was run adjusted for age, gender, duration of orthodontic treatment, type of maloclussion, type of treatment (extraction or non-extraction treatment), type of respiration (oral or nasal respiration) as possible covariates. The data were analyzed by the Statistical Package Social Sciences (version 19) software (SPSS, Chicago, IL, USA) and p-values less than 0.05 were considered significant.

3. Results

3.1. Distribution of metals in human hair from control and orthodontic groups

The concentrations of Cr, Cu, Fe, Mn and Ni levels in human hair were fitted to typical statistical distributions (Gaussian, log normal, gamma) and were shown in Fig. 1. All elements were log normal distributed, and because their values did not follow a normal distribution, all further statistical computations were based on non-parametric techniques. Table 1 shows the medians, ranges, 5–95th percentiles of the data for each element in addition to mean and standard deviation (sd) for both groups, control and orthodontic patients. Mean values (±sd) are given to make comparisons possible with other studies. Values were dispersed in control and orthodontic patients. The lower levels were found for Mn, Cr and Ni whose medians were 0.23, 0.36 and 0.36 μg/g, respectively in control group; in the orthodontic group the following medians were obtained, 0.42, 0.33 and 0.33, respectively. Fe and Cu were the predominant elements with medians about 25–30 μg/g in control and orthodontic groups (Table 1). When orthodontic and control groups were compared, only a significant increase of Mn levels was observed ($p < 0.05$).

3.2. Influence of individual factors: sex and age

We have considered the influence of two individual factors on the metals levels found in human hair from our population

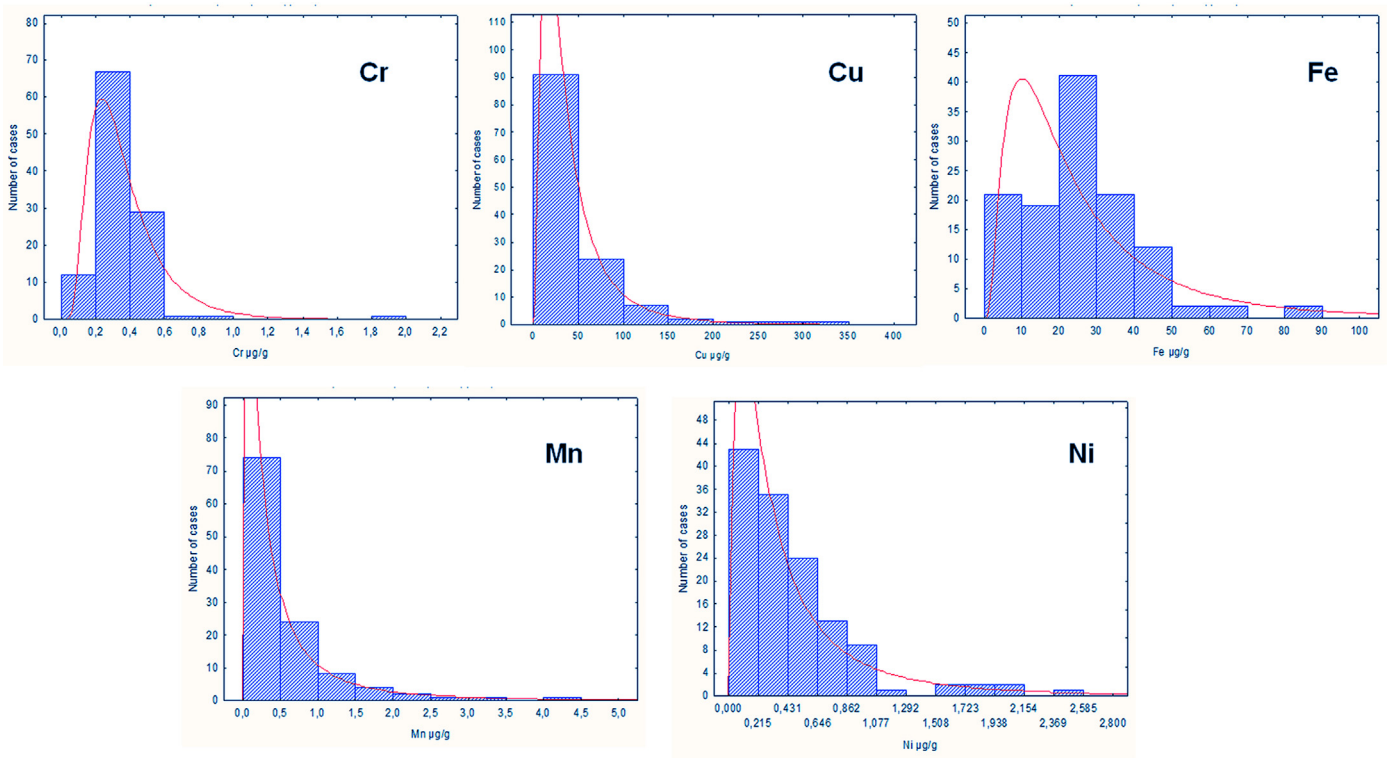

Fig. 1 – Log-normal distributions of metal concentrations found in human hair.

Table 1 – Metal levels in hair samples from the control group ($n = 56$) and orthodontic group ($n = 70$) studied.

Metals (μg/g)	Control group				Orthodontic group			
	Mean ± SD	Range	Median	5–95th percentiles	Mean ± SD	Range	Median	5–95th percentiles
Cr	0.36 ± 0.08	0.10–0.52	0.36	0.21–0.51	0.35 ± 0.26	0.04–1.86	0.33	0.05–0.65
Cu	39.07 ± 28.37	5.00–127.00	33.00	9.00–102.00	43.61 ± 57.28	5.00–340.00	24.00	7.00–158.00
Fe	27.24 ± 17.06	2.20–87.12	25.30	3.08–58.96	24.97 ± 15.00	1.76–80.08	24.86	1.98–45.76
Mn	0.36 ± 0.37	0.006–1.810	0.23	0.03–1.06	0.70 ± 0.83	0.01–4.24*	0.42	0.04–2.12
Ni	0.47 ± 0.37	0.008–1.810	0.36	0.06–1.13	0.46 ± 0.50	0.024–2.40	0.33	0.04–1.90

* The significant level is $p < 0.05$ in comparison to control group.

study. Data for the bivariate analysis among metal concentrations in relation to sex and age are presented in Tables 2 and 3, respectively, in control and orthodontic patients. In general, females had higher metal levels in both groups (control and orthodontic) analyzed (Table 2). Specifically, in the control group, females had significant ($p < 0.05$) higher levels of all metals, being these differences even more significant in Ni content ($p < 0.001$) (Table 2). The differences in the median values were markedly larger in females for Cu ($p < 0.05$), Mn and Ni ($p < 0.001$) in the test group of patients. Between groups only significant differences ($p < 0.05$) were found in Cr (decreased) and Mn (increased) contents in females.

In order to investigate significant differences related to age, two groups were considered, namely <20, and >20 years. Table 3 shows the median metal concentration in scalp hair adjusted for age. No significant associations were found between age and metal levels in the control group. Interestingly, the orthodontic treatment increased significantly Mn levels ($p < 0.05$) in young patients (<20 years old) when compared with control group.

3.3. *Interrelations between elements in human hair*

We built a data matrix and in order to statistically prove the observed correlations between the contents of the studied elements in hair, the non-parametric technique of Spearman (Miller and Miller, 2002) was applied. The most important correlations (with a probability (P) of error level less than 0.05 are the following: Cr/Cu ($r = 0.26$), Cu/Mn ($r = 0.38$), Cu/Ni ($r = 0.41$), and Mn/Ni ($r = 0.22$). All correlations were positive (levels increase or decrease similarly, in the same direction) and the sequence of correlation with Cu was: Ni > Mn > Cr.

Although typical linear correlations were inappropriate due to the non normality of the distributions of metals studied, from a pictorial viewpoint, the element-to-element linear regression plots for statistically significant correlations are shown in Fig. 2. The analysis of correlation coefficients shows that there is significantly positive correlation ($p < 0.05$) between Cu/Mn, Cu/Ni, and Fe/Ni.

3.4. *Multiple linear regression analysis of the influence of orthodontic treatment variables on the ion concentrations*

Correlation matrix only provides information about relations between two metallic elements. Nevertheless, the interactions inside humans are of more complex nature. We might expect that the level of a given element in hair should be influenced by the exposure to several other clinical factors; therefore, the relationships among different elements and critical clinical factors was examined, are better presented by a stepwise linear multiple regression analysis (Chojnacka et al., 2005) adjusted for variables described above (Table 4). The regression model showed that recorded orthodontic clinical factors did not influence the hair concentrations of Mn, Fe, Cr, Ni and Cu. As shown in Table 4, none of the recorded variables could serve as predictors of observed results [$AdjR^2$: Mn: −0.023; Cr: 0.001; Ni: 0.026; Fe: 0.022; Cu: −0.030 ($p > .05$)].

3.5. *Coefficients α and β in hair of patients*

According to Mikulewicz et al. (2011) the following coefficients α_{hair} and β_{hair} in the assessment of bioavailability of released metal ions into the organism of a patient were evaluated, being $\alpha_{hair} = C_{with\ apply}/C_{without\ apply}$ and the difference (expressed as mg/kg), β_{hair}: $C_{with\ apply} - C_{without\ apply}$

Thus, the coefficient α_{hair} is a measure of concentration factor, and β_{hair} is the difference between the content of elements in orthodontic patients *vs* control. In this study, and taking into account the previous significantly different results, the values of these coefficients were obtained for all metals considering the whole population, and specifically for Mn and Cr in relation to sex, and for Mn in relation to age (Table 5). The α_{hair} and β_{hair} values obtained confirm marked concentrations of Mn in the orthodontic patients when the whole population is considered (94.4%), also higher contents of Cu (11.6%), and lower values of Cr, Fe and Ni with respect to control group. If the gender is considered, the α_{hair} indicated that Mn levels were higher in the orthodontic group (≈85%) for females and males when orthodontic and control groups are compared. In the case of Cr, a decrease and an increase for females and males, respectively, were observed when compared orthodontic and control groups. There were variations in Mn levels with age: young people (<20 years old) in the orthodontic group showed near 3-fold higher Mn contents than the control group, whereas 1.24 increase was obtained for the >20 years group. These differences were also confirmed with the β_{hair} coefficients.

4. Discussion

Metal release from orthodontic appliances has been reported increasing the concern about the potential consequences for health of patients (Danaei et al., 2011). In this matter, several authors have evaluated the accumulation of these metals in

Table 2 – Metal levels in human hair samples and their relation with the sex.

Metals (μg/g)	Control group ($n = 56$)				Orthodontic group ($n = 70$)			
	Male ($n = 24$)		Female ($n = 32$)		Male ($n = 24$)		Female ($n = 46$)	
	Mean ± SD (median)	Range	Mean ± SD (median)	Range	Mean ± SD (median)	Range	Mean ± SD (median)	Range
Cr	0.34 ± 0.09 (0.33)	0.10–0.47	0.39 ± 0.07* (0.37)	0.21–0.52	0.39 ± 0.35 (0.33)	0.04–1.86	0.32 ± 0.14# (0.33)	0.05–0.65
Cu	28.70 ± 24.32 (19.36)	5.72–101.64	45.60 ± 29.50* (42.24)	4.84–127.00	22.81 ± 29.08 (14.96)	4.84–158.40	59.05 ± 67.58* (40.70)	6.16–339.68
Fe	21.39 ± 11.73 (19.14)	3.08–40.26	30.55 ± 18.82* (27.83)	2.20–87.12	22.23 ± 13.95 (24.86)	1.76–69.67	26.58 ± 15.51 (25.41)	1.76–80.08
Mn	0.23 ± 0.29 (0.13)	0.01–1.27	0.44 ± 0.41* (0.33)	0.04–1.81	0.45 ± 0.89 (0.14)	0.01–4.24	0.82 ± 0.77***,# (0.65)	0.02–3.29
Ni	0.22 ± 0.18 (0.16)	0.01–0.77	0.63 ± 0.38*** (0.55)	0.13–1.81	0.30 ± 0.51 (0.13)	0.02–2.05	0.54 ± 0.47*** (0.43)	0.05–2.40

* $p < 0.05$ significant level observed between male and female.
*** $p < 0.001$ significant level observed between male and female.
\# $p < 0.05$ significant level between control and orthodontic group.

Table 3 – Metal levels found in human hair and their relation with age.

Metals (μg/g)	Control group ($n = 56$)				Orthodontic group ($n = 70$)			
	<20 years old ($n = 23$)		>20 years old ($n = 33$)		<20 years old ($n = 42$)		>20 years old ($n = 28$)	
	Mean ± SD (median)	Range	Mean ± SD (median)	Range	Mean ± SD (median)	Range	Mean ± SD (median)	Range
Cr	0.38 ± 0.11 (0.37)	0.10–0.52	0.35 ± 0.06 (0.35)	0.25–0.49	0.32 ± 0.15 (0.34)	0.04–0.65	0.40 ± 0.37 (0.33)	0.05–1.86
Cu	45.02 ± 34.04 (36.52)	10.12–127.00	34.36 ± 23.83 (30.36)	4.84–113.08	44.68 ± 48.47 (28.60)	6.16–292.16	43.23 ± 72.25 (16.28)	4.84–339.68
Fe	29.49 ± 18.16 (28.16)	5.06–87.12	25.08 ± 16.03 (23.10)	2.20–65.12	24.61 ± 15.09 (24.64)	1.76–80.08	25.67 ± 14.38 (25.98)	1.76–69.67
Mn	0.28 ± 0.24 (0.27)	0.02–1.06	0.41 ± 0.44 (0.22)	0.01–1.80	0.81 ± 0.95# (0.47)	0.01–4.24	0.51 ± 0.50 (0.33)	0.08–2.12
Ni	0.38 ± 0.24 (0.35)	0.01–0.95	0.53 ± 0.44 (0.43)	0.02–1.81	0.49 ± 0.45 (0.38)	0.04–2.05	0.42 ± 0.59 (0.26)	0.02–2.40

\# The significant level observed was $p < 0.05$ when control and orthodontic groups were compared.

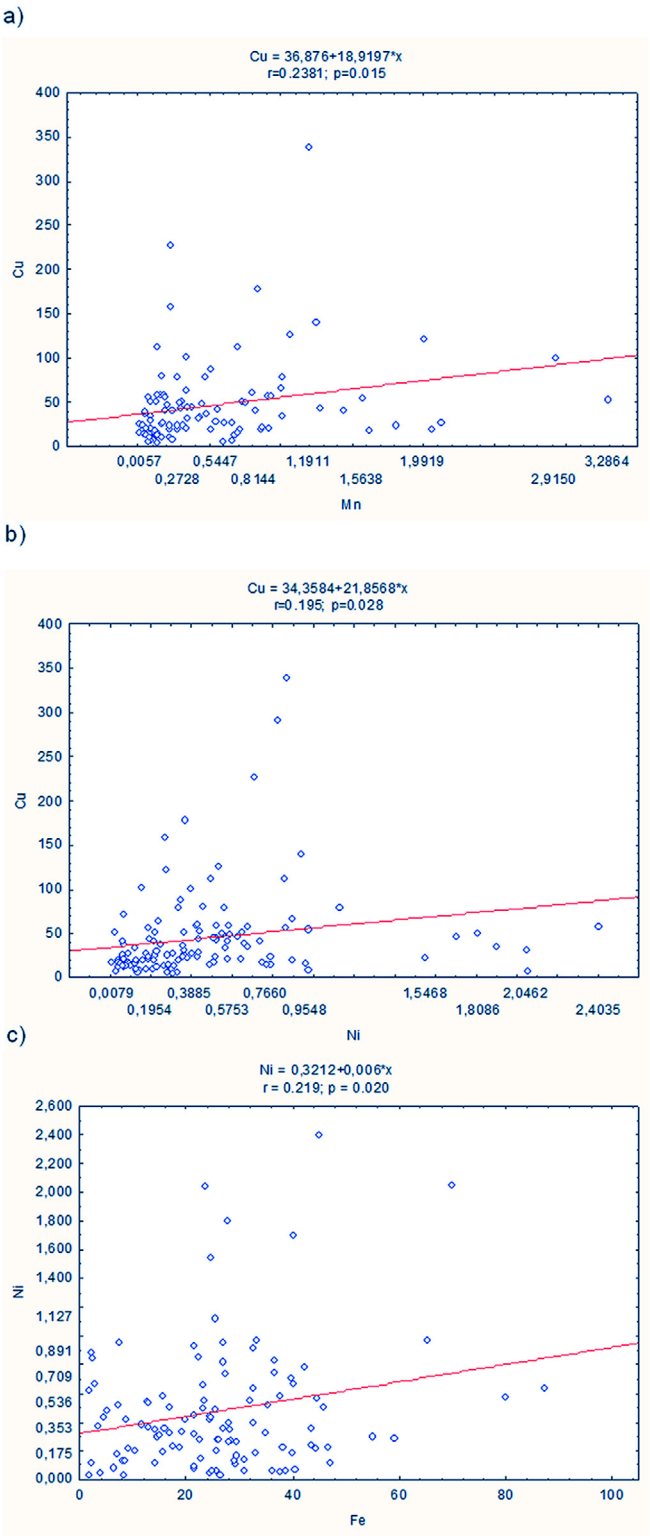

Fig. 2 – Interrelation between elements determined en human hair: (a) Mn/Cu; (b) Ni/Cu and (c) Fe/Ni.

different matrices, such as saliva (Eliades et al., 2003; Fors and Persson, 2006; Petumenou et al., 2009; Sahoo et al., 2011), and more recently, mucosa cells (Martin-Cameán et al., 2014). The determination of trace elements in human hair has attracted the attention of investigators for at least 50 years, and it is considered one of the best choices to study environmental and occupational exposure (Wolowiec et al., 2013). However, regarding orthodontic patients, only Mikulewicz et al. (2011) have performed a preliminary study evaluating the exposure to metals released from fixed orthodontic appliances in the patients' hair.

The elemental composition of hair (unlike blood or urine) reflects long-term exposure to these metals, since hair is an indicator of past changes in metabolism and environmental exposure (Chojnacka et al., 2005). Hair content depends on age, sex (Senofonte et al., 2000; Chojnacka et al., 2006a), dietary and living habits (Miekeley et al., 1998; Chojnacka et al., 2006b), and exposure (associated with urbanization and industrialization) (Ashraf and Jaffar, 1997; Gil et al., 2011), *etc.* Moreover, it is significant to take into account the geographical location of the population (Amaral et al., 2008). All these factors can explain the variability in the reference values reported in the scientific literature. In this work, we have determined for the first time Cr, Cu, Fe, Mn and Ni contents in hair of a wide population in our country, after undergoing orthodontic treatment, and significant increases of Mn content in patients were found in comparison to control group. Moreover, differences related to sex and age have been firstly reported not only in the control group but also in the orthodontic group.

Metal levels found in our study population presented high variation (Table 1), similarly to those reported in the scientific literature. Table 6 presents ranges reported by various authors, because internationally accepted reference ranges for the content of these metals have not been established. Recently, Mikulewicz et al. (2013) have performed a systematic review of reference values of elements in human hair and indicated that more work is this field is required. The reason of the lack of reference values is the absence of clear guidance on the methodology for the selection of the population, sampling, their preparation and digestion, as well as analysis. In comparison to Mikulewicz et al. (2011), higher Cr and Fe contents have been found, whereas Mn and Ni values were similar; Cu was not determined previously. In our study, a significant increase of Mn was found in orthodontic patients, whereas no differences between exposed and non-exposed groups were reported by Mikulewicz et al. (2011), may be due to the small number of participants included.

Literature reports focus mainly on the release of Ni and Cr from orthodontic appliances Chromium can cause hypersensivity and dermatitis, and induce cytotoxicity and genotoxicity (International Agency for Research on Cancer, IARC, 1990; Burrows, 1986). In relation to other values reported, our Cr values are very similar to those found by Grabeklis et al. (2011) for a non exposed-population, and slightly lower than those from workers of a chemical plant. Cr values reported by Chojnacka et al. (2005) in urban population from Poland, or by Afridi et al. (2006) for a non-exposed population were higher, whereas those reported by Dongarrà et al. (2012) were lower than ours. These differences could be related to the geological characteristics of the study areas, although, at the moment, the

Table 4 – Forward stepwise multiple linear regression analysis of metal ion concentration detected in the patient' hair.

Metal ion	$adjR^2$	F	p-Value	Predictor	B	p-Value	Partial correlation
Cr	0.001	1.015	0.394	Treatment time	−.033	.303	−.150
				Maloclussion	−.008	.886	−.021
				Extraction treatment	−.127	.144	−.212
Cu	−0.030	0.291	0.832	Treatment time	−2.729	.577	−.067
				Maloclussion	−5.770	.564	−.070
				Extraction treatment	.901	.949	.008
Fe	0.022	0.431	0.731	Treatment time	−1.544	.338	−.127
				Maloclussion	2.108	.485	.093
				Extraction treatment	1.895	.648	.061
Mn	−0.023	0.587	0.626	Treatment time	.051	.611	.072
				Maloclussion	.059	.739	.047
				Extraction treatment	−.225	.374	−.125
Ni	0.026	1.543	0.213	Treatment time	−.025	.589	−.071
				Maloclussion	.134	.177	.177
				Extraction treatment	.251	.063	.242

Table 5 – Coefficients α_{hair} and β_{hair} in orthodontic and control groups (whole population) for all metals, and in the case of differences in metal content in relation to sex (Cr, Mn) and age (Mn).

Metals	Whole population		In relation to sex				In relation to age			
			Female		Male		<20 years old		>20 years old	
	α_{hair}	β_{hair} (μg/g)	α_{hair}	β_{hair} (μg/g)	α_{hair}	β_{hair} (μg/g)	α_{hair}	β_{hair} (μg/g)	α_{hair}	β_{hair} (μg/g)
Cr	0.972	−0.0123	0.827	−0.0666	1.175	0.0587				
Cu	1.116	4.5400								
Fe	0.917	−2.2670								
Mn	1.944	0.3411	1.889	0.3878	1.859	0.2060	2.872	0.5252	1.243	0.0992
Ni	0.979	−0.0066								

supposed association between metal profiles and geographic area should be taken with caution (Dongarrà et al., 2012). In contrast, hair levels found by Gil et al. (2011) in workers exposed to the metal were 10-fold higher than our results.

In the literature, initially most papers have been dedicated mainly to the detection of Cu and Zn in human hair because they are the most abundant elements present (Sturaro et al., 1994). The mean Cu results obtained in this study were similar to those found by Miekeley et al. (1998) in a population from Brazil. In contrast, the values reported by other authors (Ward and Cambell, 1986; Ward et al., 1987; Sturaro et al., 1994; Nowak, 1998; Chojnacka et al., 2005; Dongarrà et al., 2012) were lower than ours. Similarly to Dongarrà et al. (2012) which measured in children's hair nineteen elements, copper was one of the most abundant elements found in our study. The second mayoritary element was Fe, whose levels were within the wide ranges reported by Kamakura (1983), Ward and Cambell (1986), Ward et al. (1987), and Senofonte et al. (2000). Therefore, our Fe levels found do not exceed those previously reported for non-exposed populations. Both predominant elements are generally accepted as essentials, with potential for toxicity. Copper is a component of all living cells and is associated with many oxidative processes, but in cases of Wilson's disease the low level of ceruloplasmin results in excessive accumulation. Iron takes part in oxygen transport and utilization, although chronic iron toxicity or Fe overload in adults is a common problem. There is epidemiologic evidence for a relationship between Fe levels and cardiovascular disease, and it may contribute to lipid peroxidation in an early step in the formation of atherosclerotic lesions (Goyer and Clarkson, 2001).

Nickel is a toxic metal, widely used in various industrial processes and the level of exposure is frequently related to the degree of urbanization of the assayed population (Jergovic et al., 2010). One of the roles of Ni in the alloys employed in orthodontic appliances is to increase the strength, ductility, and resistance to general, crevice and erosion corrosion. However, it has been reported that the major corrosion products are Fe, Cr, and Ni for stainless steel and Ti and Ni for nickel–titanium alloys (Amini et al., 2008). Among stainless steel and nickel–titanium corrosion products, Ni and Cr have received the most attention because of their reported adverse effects. Nickel is a known allergen, and adverse reactions related to Ni-containing orthodontic devices such as archwires, brackets, and soldered stainless steel face-bows have been reported (Dunlap et al., 1989; Greppi et al., 1989). Moreover it has carcinogenic and mutagenic effects (IARC, 1990). According to Sturaro et al. (1994) the analysis of Ni in hair was a good indicator of long-term exposure, and they studied the exposure to Ni of steel mill workers and found that the content of Ni in hair in the control group was 5.25 mg/kg and increased significantly in workers with the time of employment. Mikulewicz et al. (2011) reported in hair of control group Ni values of 0.364 ± 0.248, and higher but not significant values in orthodontic patients (0.507 ± 0.372), although these authors indicated that the release of Ni from orthodontic appliances can constitute a significant resource of exposure of this

Table 6 – Values in scalp hair of metal compounds selected in this study and reported in the literature.

Reference values	*n*	Cr	Cu	Fe	Mn	Ni	Notes
Present study (μg/g)	56	0.36 ± 0.08	39.07 ± 28.37	27.24 ± 17.06	0.36 ± 0.37	0.47 ± 0.37	Control group
	70	0.35 ± 0.26	43.61 ± 57.28	24.97 ± 15.00	0.70 ± 0.83	0.46 ± 0.50	Orthodontic group (mean ± SD)
Mikulewicz et al. (2011) (μg/g)	18	0.129 ± 0.131		11.74 ± 8.07	0.485 ± 0.491	0.364 ± 0.248	Control group
	28	0.133 ± 0.088		12.22 ± 9.91	0.574 ± 0.620	0.507 ± 0.372	Orthodontic group
Husain et al. (1980) (μg/g)	102	2.45 ± 1.30				1.25 ± 0.72	Control (male)
Kamakura (1983) (μg/g)	1899	0.20–0.77	6.0–69.1	5.5–87.4	0.06–4.51	0.17–3.00	Control males/females No environmental or occupational exposure to metals
Ward and Cambell (1986)	32	0.56–1.92	3.42–8.12	18.4–52.8	0.57–1.68	1.62–4.58	Patients on maintenance with lithium carbonate
Ward et al. (1987)	36	0.20–1.02	7.2–19.4	12.9–96.4	0.20–4.30	0.55–3.59	Control males/females
Leotsinidis and Kondakis (1990) (μg/g)	75	0.61/0.56				0.82/1.16	Control males/females
Kono et al. (1990)	237					0.52 ± 0.28	Control
						0.66 ± 0.27	Workers
Gammelgaard and Veien (1990) (μg/g)	16					0.149	Non-exposed
	64					0.340	Hypersensitive people (median value)
Sturaro et al. (1994) (μg/g)	132		22 ± 10 (males) 24 ± 10 (females)		0.6 ± 0.5 (males) 0.7 ± 0.5 (females)	1.6 ± 2.6 (males) 2.8 ± 5.2 (females)	Control males/females no subjected to any chemical treatment
Bencko (1995) (μg/g)	62/102	0.124				0.286	Control
Leung and Huang (1997) (μg/mL)	12–17	0.08 ± 0.11			2.34 ± 2.30	0.61 ± 0.26	Healthy people (30–50 years old)
Bermejo-Barrera et al. (1998) (μg/g)	25				0.03 ± 1.20		Healthy people (range)
Miekeley et al. (1998) (μg/g)	1091	<0.30	44.1 ± 3.5	20.8 ± 2.2	5.0 ± 0.5	0.7 ± 0.1	Rio Janeiro city (Brazil)
Nowak (1998) (μg/g)	266	0.60 ± 1.13	7.96 ± 9.12	45.7 ± 37.7	2.41 ± 2.24	0.75 ± 1.15	Non-industrialized region from Poland (Mean ± SD)
Luse et al. (2000) (μg/g)	46				1.83 ± 0.18		Control
					5.78 ± 1.60		Welders
Rodushkin and Axelssom (2000) (μg/g)	114	0.167 ± 0.118		9.6 ± 4.4	0.560 ± 0.550	0.430 ± 0.400	Urban population from Sweden (Mean ± SD)
Senofonte et al. (2000) (μg/g)	160–412	0.08–4.56 (*n* = 160)	7.2–82.7 (*n* = 412)	5.9–36.8 (*n* = 408)	0.04–0.77 (*n* = 378)	0.07–3.40 (*n* = 263)	School children (boys) (5th-95th values)
Schamberger (2003) (μg/mL)	50	0.06 ± 0.17			0.62 ± 1.70	0.47 ± 0.31	Control
Goullé et al. (2005) (μg/g)	45	0.20			0.067	0.23	Healthy people (median values)
Chojnacka et al. (2005) (μg/g)	83	0.57 ± 1.04	12.35 ± 12.05	15.00 ± 16.07	0.60 ± 0.59	0.84 ± 1.13	Urban population from Poland
Afridi et al. (2006) (μg/g)	75	3.82 ± 0.67				5.25 ± 1.46	Non-exposed
	56	5.86 ± 2.21				3.43 ± 1.16	Workers steel
Moreda-Piñeiro et al. (2007) (μg/g)	10					5.95 ± 4.30	Non-exposed
Rodrigues et al. (2008) (μg/g)	280				1.3 ± 2.4 (0.05–6.7)		Healthy Brazilian adults (range)
Chojnacka et al. (2010)	117	0.91–1.53	8.51–34.97	16.9–29.6	0.459–1.046	0.508–1.534	Poland - Urban area (Percentile 10 and 90)
Grabeklis et al. (2011) (μg/g)	262	0.369 ± 0.017			0.934 ± 0.065	0.360 ± 0.028	Non exposed
		0.487 ± 0.043			1.34 ± 0.18	0.459 ± 0.057	Workers exposed chemical plant

Table 6 – (Continued)

Reference values	*n*	Cr	Cu	Fe	Mn	Ni	Notes
Gil et al. (2011) (μg/g)	178	3.43 ± 7.93			2.35 ± 4.66	3.29 ± 16.59	Mean ± SD values for workers
		0.99			1.19	0.71	Median values for workers
Dongarrà et al. (2012)	336	Urban area	Urban area		Urban area	Urban area 0.420	Median values of Children (11–13
		(*n* = 131) 0.075	20.0		0.268	Industrial area	years old) from Sicily
		Industrial	Industrial		Industrial	0.400	
		area	area 11.7		area 0.350	Rural area 0.740	
		(*n* = 58) 0.139	Rural area		Rural area		
		Rural area	17.0		0.400		
		(*n* = 47) 0.260					
Vanaelst et al. (2012)	218		10.1–46.6	3.66–17.27			P10–P90 Belgian school girls
Vanaelst et al. (2013)	140		18	7			Median values of school girls

element. By contrast, in our study, similar contents were obtained in control (0.47 ± 0.37) and orthodontic patients (0.46 ± 0.50). Consequently, our results are not consistent with this hypothesis, and since our values are similar to others found in the scientific literature for non-exposed populations (Kono et al., 1990; Rodushkin and Axelssom, 2000; Schamberger, 2003; Grabeklis et al., 2011; Dongarrà et al., 2012) the values obtained cannot be considered of health concern.

Manganese is a transitional and essential metal, and is a cofactor for a number of enzymatic reactions, particularly those involved in phosphorylation, cholesterol, and fatty acids synthesis. Mn is present in all living organisms. There is current interest in the toxicology of manganese because of potential exposure from the use of Mn-containing fuel additives, and because its industrial use has also expanded in recent years (as a ferroalloy in the iron industry and as a component of alloys used in welding) (Goyer and Clarkson, 2001). There are few reported cases of Mn toxicity from oral ingestion, because homeostatic mechanisms are involved for excreting its excess. The oral absorption of Mn is increased by Fe deficiency, which may contribute to variations in individual susceptibility (Goyer and Clarkson, 2001). In our study, Mn is the only element whose contents in human hair were significantly increased in orthodontic patients (0.70 ± 0.83 μg/g), in comparison to control group (0.36 ± 0.37 μg/g). This result is consistent with Mikulewicz et al. (2011) which also found an increase (18%) of this metal in orthodontic patients, although not statistically significant. Danaei et al. (2011) demonstrated *in vitro* Mn release from orthodontic brackets kept in different mouthwashes. Also, *in vitro* Mikulewicz et al. (2011) found 68 μg/L of Mn from orthodontic appliances composed of different alloys incubated in artificial saliva, although these authors suggested that the only possible risk of exposure would be nickel. Our results obtained in the control group are in agreement with data reported by Dongarrà et al. (2012) in children living in urban or industrial areas in Sicily. Moreover, the mean values for the orthodontic group were similar to those reported by several studies in the literature for urban or control populations (Sturaro et al., 1994; Chojnacka et al., 2005; Grabeklis et al., 2011), and were much lower than values found for workers exposed to the element (Luse et al., 2000; Gil et al., 2011). Thus, these values cannot be considered of health concern.

Metal hair content also varies according to sex and age (Sturaro et al., 1994; Leung and Huang, 1997). Higher metal levels in hair were found for females in comparison to males in the present study. Other reports have also confirmed that sex is an important covariate influencing trace-element content of human scalp-hair with females showing higher values (Moon et al., 1986; Leotsinidis and Kondakis, 1990; Sturaro et al., 1994; Senofonte et al., 2000; Gil et al., 2011). In addition, other clinical variables potentially associated with observed metallic concentrations were explored for the first time in literature. Nevertheless, as shown in Table 4, all selected predictors could just explain a slight influence on the overall concentration of these metallic ions.

We have considered two different age groups, whose choice allowed the estimation of the concentration of these elements in two different moments of human life: childhood and adult age. In general the results obtained suggest that the metals concentrations are not dependent on age, with

the exception of Mn levels, which decreased with aging in the orthodontic group. Sturaro et al. (1994) considered the influence of color, age and sex on the content of several metals in human hair, and found that Cu concentrations decreased with increased age. Moreover, Mn increased in blond and decreased in brown hair with aging, independently of sex. Senofonte et al. (2000) found significant differences of the element content in hair among the three age groups considered in children (3–6, 7–10 and 11–13 years). Some elements showed a tendency to increase with age (Cu in female, Co, Zn), whereas there was little variations in others (Fe, Mn). Chojnacka et al. (2006a) found that the levels of Mn, Ni, Ti, Se, Hg, Cd, As and Co were not a function of age.

The studies on inter-element interactions in hair are scarce. Rodushkin and Axelssom (2000) reported synergistic interactions between several elements, although the authors found no significant correlations for essential elements (Zn, Cu, Se) and potentially toxic (Cd, Pb, As and Tl). Later, Chojnacka et al. (2005) studied inter-element interactions in human hair sampled from a population group living in urbanized and industrialized region of Poland. They found strong positive correlations between Ca, Mg, Mn and Sr, and all these elements were negatively correlated with another group (Pt, W). A correlation Fe–Mn has been also reported, and according to Chojnacka et al. (2006b) these correlations resulted either from the common occurrence of elements or synergistic effects between them. In the case of orthodontic patients, Mikulewicz et al. (2011) reported statistically significant correlation only between Fe and Cr (synergism). In our study, the positive correlations found between Cu/Mn, Cu/Ni an Fe/Ni showing a mutual dependence of these metals in hair, probably are due to similar chemical nature: Fe and Ni lie in the same VIIIb group in periodic table of elements, Cu belongs to Ib group, and Mn to VII group.

5. Conclusions

Our data altogether indicate that hair mineral analysis is a good method to investigate the release of the elements from fixed orthodontic appliances, and to evaluate the bioavailability of toxic metals. Moreover this matrix may be used as additional and/or alternative samples to saliva or mucosa cells in order to know long-term exposure to metals from orthodontic treatments. In patients, differences in the content of metals in hair were only significantly increased for Mn when compared to the control group, but their levels were of the same magnitude to other control populations, and consequently, no risks linked to the treatment have been found. It has been demonstrated higher accumulation of metals in hair of females, and in general the metals concentrations are not dependent on age, with the exception of Mn levels, which decreased with aging in the orthodontic group compared to the control group. The positive correlations found between Cu/Mn, Cu/Ni and Fe/Ni showing a mutual dependence of these metals in hair, probably are due to similar chemical nature. This study provides values that may be useful for future comparative purposes with other studies focused on hair mineral analysis in patients undergoing fixed appliances treatment, and it would be worthwhile carrying out further studies on larger control and experimental groups and collecting more information about the potential health effects derived from their accumulation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgement

The authors wish to thank the Junta de Andalucía (PAIDI CTS-358) for the financial support for the present study. The present article constitutes part of the doctoral thesis of Martín-Cameán A at the University of Seville, Spain.

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CAPÍTULO 4 / CHAPTER 4

Ana Martín-Cameán, Ángeles Jos, María Puerto, Ana Calleja, Alejandro Iglesias-Linares, Enrique Solano, Ana M. Cameán

IN VIVO DETERMINATION OF ALUMINUM, COBALT, CHROMIUM, COPPER, NICKEL, TITANIUM AND VANADIUM IN ORAL MUCOSA CELLS FROM ORTHODONTIC PATIENTS WITH MINI-IMPLANTS BY INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (ICP-MS)

Journal of Trace Elements in Medicine and Biology (en segunda revisión) **2015**

Elsevier Editorial System(tm) for Journal of Trace Elements in Medicine and Biology
Manuscript Draft

Manuscript Number:

Title: In vivo determination of Aluminium, Cobalt, Chromium, Copper, Nickel, Titanium and Vanadium in oral mucosa cells from orthodontic patients with mini-implants by Inductively coupled plasma-mass spectrometry (ICP-MS)

Article Type: Original Paper

Section/Category: Toxicology

Keywords: In vivo, metals, oral mucosa cells, orthodontic, miniscrew

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Abstract: Miniscrews are used as orthodontic anchorage devices in the dentistry clinical practice but the in vivo metallic release from these structures has been not previously investigated. The aim of this study was to determine the content of Al, Co, Cr, Cu, Ni,Ti and V in oral mucosa cells of control subjects, patients under orthodontic treatment and with both, orthodontic treatment and miniscrew, in order to know the contribution of these mini-implants to the total metallic content. ICP-MS measurements revealed the following ascending order: Cr < Ni < Ti < Cu < Al, and Co and V were practically undetected. Significant differences in comparison to the control group were found for Cu in the orthodontic group, and for Ni in both, orthodontic and orthodontic+miniscrew groups. Potential correlations among metallic elements and with some clinical factors were also explored. These findings suggest that miniscrews do not increase significantly the metal release.

Cover Letter

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Dear Editor,

I would be very grateful if you consider the article entitled:

In vivo determination of Aluminium, Cobalt, Chromium, Copper, Nickel, Titanium and Vanadium in oral mucosa cells from orthodontic patients with mini-implants by Inductively coupled plasma-mass spectrometry (ICP-MS)

for its publication in the journal "Journal of Trace Elements in Medicine and Biology"**.**

The authors of the article were: Ana Martín-Cameán, Ángeles Jos, María Puerto, Ana Calleja, Alejandro Iglesias-Linares, Enrique Solano, and Ana M. Cameán

The corresponding author's is: Ana M. Cameán

Nowadays, miniscrews are used in orthodontics as auxiliary devices in order to provide extra anchorage. The in vivo metallic relase from these structures has not been previously investigated. The aim of this study was to determine the content of Al, Co, Cr, Cu, Ni, Ti and V in oral mucosa cells of control subjects, patients under orthodontic treatment and with both, orthodontic treatment and miniscrew, in order to know the contribution of these mini-implants to the total metallic content. ICP-MS measurements revealed the following ascending order: Cr < Ni < Ti < Cu < Al, and Co and V were practically undetected. Significant differences in comparison to the control group were found for Cu in the orthodontic group, and for Ni in both, orthodontic and orthodontic+miniscrew groups. Potential correlations among metallic elements and with some clinical factors were also explored. The little research in this field and the importance of this subject for the health status of orthodontic patients and the wide use of miniscrews are reasons that justify the interest of the results obtained.

Looking forward to hearing from you,

Ana M. Cameán

Highlights

In vivo metal release from orthodontic appliances and miniscrew by ICP-MS

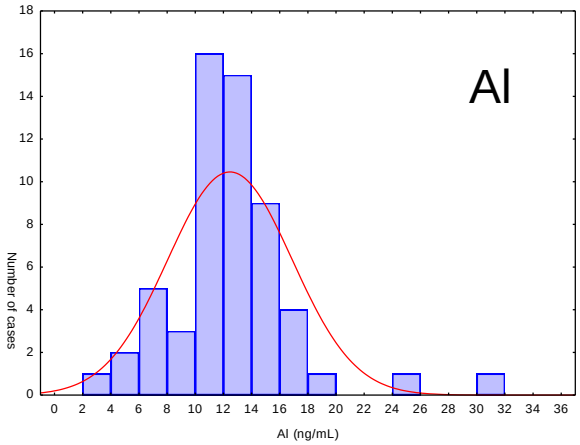
The following ascending order of metals was found in oral mucosa cells:
Cr<Ni<Ti<Cu<Al

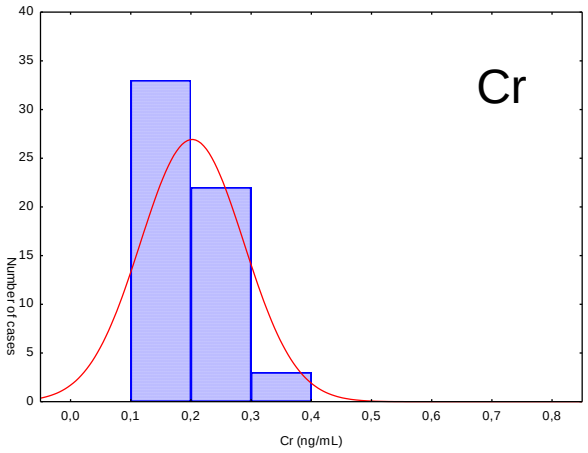
Co and V values were practically undetectable in oral mucosa cells

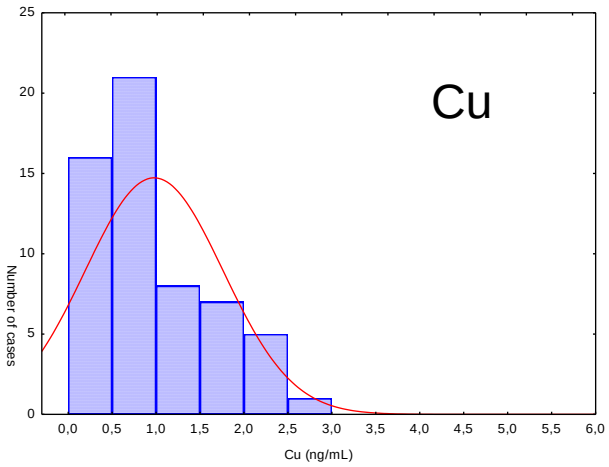
Higher Ni levels were found in orthodontic patients and with miniscrew *vs* control.

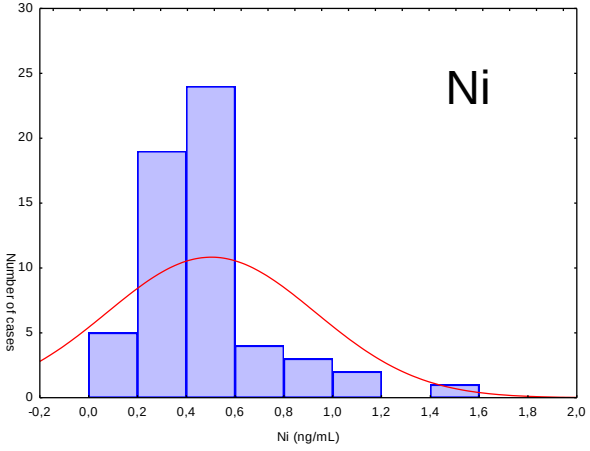
Cu values were detected in orthodontic patients in comparison to control group

Figure 1


Al
Number of cases
Al (ng/mL)


Cr
Number of cases
Cr (ng/mL)


Cu
Number of cases
Cu (ng/mL)


Ni
Number of cases
Ni (ng/mL)

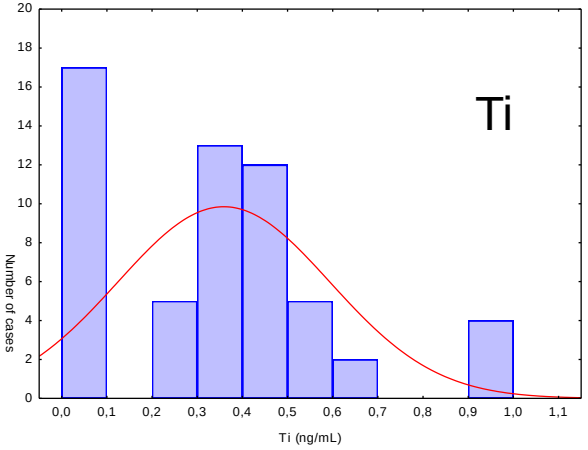

Ti
Number of cases
Ti (ng/mL)

Figure 2

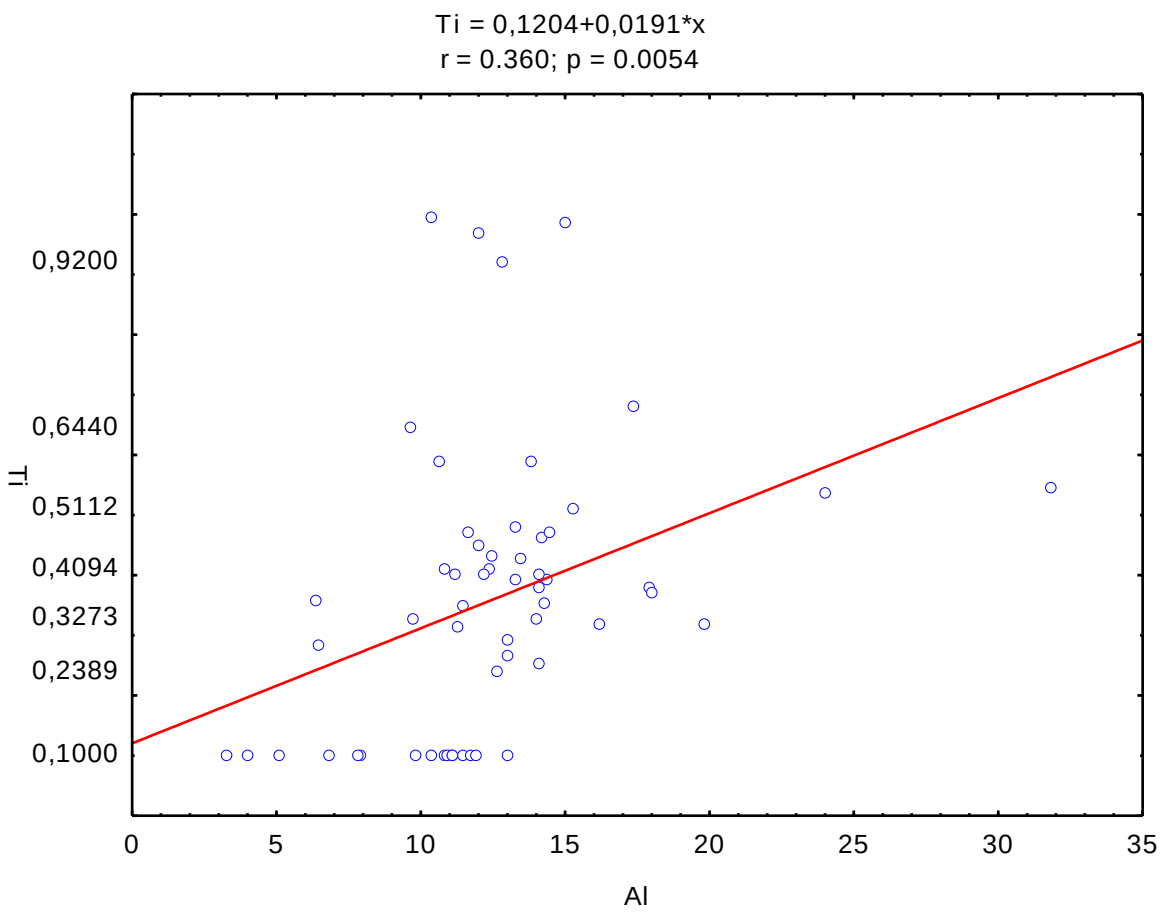

Ti = 0,1204+0,0191*x
r = 0.360; p = 0.0054
0,9200
0,6440
0,5112
0,4094
0,3273
0,2389
0,1000
Ti
0
5
10
15
20
25
30
35
Al

Table 1

Table 1. Comparison of LODs and LOQs of the present study with previous works

		Al	Ti	V	Cr	Co	Ni	Cu
LOD (ng/mL)	Martín-Cameán et al., 2014 a, b	-	0.90	2.8	0.38	0.10	0.67	0.49
	Present study	1.0	0.20	0.08	0.23	0.38	0.14	0.13
LOQ (ng/mL)	Martín-Cameán et al., 2014 a, b	-	1.80	3.4	1.13	0.20	1.81	0.98
	Present study	3.83	0.6	0.23	0.67	0.90	0.41	0.39

Table 2

Table 2. Metal levels in oral mucosa cells from the control group (n = 20), orthodontic group (n=20) and orthodontic+miniscrew group (n=20) studied.

Metals (ng/mL)	**CONTROL GROUP**				**ORTHODONTIC GROUP**				**ORTHODONTIC+MINISCREW GROUP**			
	Mean ± SD	**Range**	**Median**	**5-95th percentiles**	**Mean ± SD**	**Range**	**Median**	**5-95th percentiles**	**Mean ± SD**	**Range**	**Median**	**5-95th percentiles**
Al	12.50 ± 3.37	5.12-19.81	12.34	5.12-19.81	12.21 ± 2.19	7.89-18.00	11.96	8.78-16.50	12.70 ± 6.74	3.30-31.85	12.16	3.30-31.85
Cr	0.19 ±0.04	0.12-0.27	0.18	0.12-0.27	0.19 ± 0.05	0.12-0.30	0.18	0.12-0.27	0,21 ± 0.07	0.12-0.32	0.23	0.12-0.32
Cu	0.71 ± 0.39	0.29-1.60	0.57	0.29-1.61	1.13 ±0.69*	0.14-2.30	0.89	0.24-2.28	1,054 ± 0.71	0.07-2.67	0.93	0.07-2.67
Ni	0.34 ±0.21	0.07-0.89	0.26	0.07-0.89	0.53 ± 0.17*	0.28-0.91	0.48	0.33-0.89	0.55 ± 0.32*	0.23-1.46	0.45	0.23-1.46
Ti	0.34 ± 0.12	0.10-0.51	0.38	0.10-0.51	0.40 ± 0.34	0.10-1.00	0.28	0.10-0.99	0.33 ± 0.19	0.10-0.68	0.35	0.10-0.68

* The significant level is $p < 0.05$ in comparison to control group

Table 3. Forward stepwise multiple linear regression analysis of metal ion concentration detected in the patient´s mucosa cells .

Metal ion	*adj*R^2	F	*p*-value	Predictor	B	*p*-value	Partial correlation
Al	0.205	1.889	0.108	Miniscrew	-1.601	.259	-.240
				Gender	.643	.627	.105
				Type of braces	-1.927	.173	-.287
				Upper archwire	1.506	.022	.467
				Lower archwire	.935	.438	.166
				Cr	1.750	.875	.034
				Ni	3.172	.199	.272
				Cu	.881	.320	.212
				Ti	4.197	.059	.390
Cr	0.160	1.654	0.161	Miniscrew	.046	.089	.354
				Gender	.030	.231	.254
				Type of braces	.008	.775	.062
				Upper archwire	-.015	.256	-.242
				Lower archwire	.021	.366	.193
				Al	.001	.875	.034
				Ni	.005	.923	.021
				Cu	.028	.094	.349
				Ti	-.003	.945	-.015
Ni	-0.072	0.769	0.646	Miniscrew	.177	.140	.311

				Gender	.069	.542	.131
				Type of braces	.076	.540	.131
				Upper archwire	-.061	.303	-.220
				Lower archwire	.023	.822	.048
				Al	.023	.199	.272
				Cr	.092	.923	.021
				Cu	-.052	.498	-.145
				Ti	.121	.541	.131
Cu	-0.001	0.996	0.471	Miniscrew	-.091	.791	-.057
				Gender	-.297	.346	-.201
				Type of braces	-.021	.952	-.013
				Upper archwire	-.114	.494	-.147
				Lower archwire	.205	.482	.151
				Al	.051	.320	.212
				Cr	4.340	.094	.349
				Ni	-.408	.498	-.145
				Ti	-.393	.478	-.152
Ti	0.083	0.311	0.287	Miniscrew	-.096	.471	-.155
				Gender	-.169	.161	-.295
				Type of braces	-.091	.496	-.146
				Upper archwire	-.074	.244	-.247

	Lower archwire	**.019**	**.865**	**.037**
	Al	**.036**	**.059**	**.390**
	Cr	**-.071**	**.945**	**-.015**
	Ni	**.142**	**.541**	**.131**
	Cu	**-.059**	**.478**	**-.152**

Al: Aluminium; Cr: Chromium; Ni: Niquel; Cu: Copper; Ti: Titanium

In vivo determination of Aluminium, Cobalt, Chromium, Copper, Nickel, Titanium and Vanadium in oral mucosa cells from orthodontic patients with mini-implants by Inductively coupled plasma-mass spectrometry (ICP-MS)

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Abstract

Miniscrews are used as orthodontic anchorage devices in the dentistry clinical practice but the *in vivo* metallic release from these structures has been not previously investigated. The aim of this study was to determine the content of Al, Co, Cr, Cu, Ni,Ti and V in oral mucosa cells of control subjects, patients under orthodontic treatment and with both, orthodontic treatment and miniscrew, in order to know the contribution of these mini-implants to the total metallic content. ICP-MS measurements revealed the following ascending order: Cr < Ni < Ti < Cu < Al, and Co and V were practically undetected. Significant differences in comparison to the control group were found for Cu in the orthodontic group, and for Ni in both, orthodontic and orthodontic+miniscrew groups. Potential correlations among metallic elements and with some clinical factors were also explored. These findings suggest that miniscrews do not increase significantly the metal release.

Keywords: in vivo, metals, oral mucosa cells, orthodontic, miniscrew

1. Introduction

Several oral clinical manifestations in orthodontic treatment are associated with corrosion products and ion release from the appliances employed [1]. Saliva acts as an electrolyte for electron and ion conduction, and the fluctuation of pH and temperature, the enzymatic and microbial activity, and the various chemicals introduced into the oral cavity through food and drink are all corrosion conductors [2]. The metal appliances used in orthodontic treatment (brackets, tubes, bands) remain in the mouth for an average of 2 years in this potentially corrosive environment. In spite of the high resistance to corrosion of the alloys used for their manufacture, they may still suffer some localized corrosion resulting from the oral cavity conditions [3]. The inherent heterogeneity of each metal alloy and its use with other alloys, the microsurface discontinuity, the forces acting on the appliances, and the friction between wires and brackets also add to the corrosion process [4].

Various studies have evaluated the discharge of metal ions from orthodontic appliances in biologic fluids, and most have concluded that they do not reach toxic concentrations [5-7]. However, it cannot be excluded that even nontoxic concentrations might be sufficient to produce biologic changes in the oral mucosa. In fact, cations released from dental alloys may cause significant biological alterations (DNA synthesis, alkaline phosphatase activity, etc) at non-cytotoxic concentrations [8]. Thus, in addition to the cytotoxic effects of metal ions, the physical and mechanical effects of orthodontic appliances could also induce changes in the oral mucosa [9]. Moreover, it has been reported that metal ions are taken up by the adjacent oral tissues [10-12]. Occasionally, the host response to the elemental release differs in the

nature and amount of the released elements. Classically allergic responses are characterized by dose-independence, this is, low doses that would not cause inflammation through toxicity but it would cause it by activating immune cells `13]. Also, mutagenicity and carcinogenic effects are not related with the dose of the toxicant. Therefore, knowledge about the elemental release from these materials into the oral cavity in regard to quantification is of great importance [14-15].

There are numerous *in vitro* studies that have demonstrated the release of metals from orthodontic alloys [1, 16-18] which have been revised by Mikulewicz et al., [19]. Recently, these authors have evaluated the release of some metals (nickel, chromium, copper) from fixed orthodontic appliances *in vitro* in a continuous- flow system [20]. However, there has been little *in vivo* research into the adverse effects of metal release, their absorption and toxicity. The few studies available involve saliva analysis [10, 21-22]. The disadvantage of saliva is related to its flow, which is influenced by many factors. Saliva flow does not influence all substance concentrations to the same degree, so it can still be a useful matrix for non-flow-dependent chemicals [23]. To the extent of our knowledge, the release of metals in oral mucosa cells, with prolonged contact with fixed appliances, has been scarcely investigated [4, 9-10, 12, 17], although this matrix shows the same advantages than saliva samples previously mentioned [24].

The release of elements from dental casting alloys in different matrices has been mainly measured using either Atomic absorption spectroscopy (AAS) [25-26], Inductively coupled plasma-optical emission spectrometry (ICP-OES) [16, 27], or Inductively coupled plasma mass spectrometry (ICP-MS) [1, 28-30]. For many elements, the power of detection of ICP-OES is not sufficient to determine elemental background

concentrations. In general, lower limits of detection (LODs) are possibly obtained by ICP-MS in comparison to ICP-AES [31]. Currently, by application of ICP-MS the fast and accurate routine multi-element determination in biological samples has become possible due to improved sensitivity and robustness. In this sense, we have recently published two validation methods to quantify some elements in oral mucosa cells using this technique [24, 32].

The development of orthodontic treatments introduced in 1997 mini-screws by Kanomi [33]. They were mainly used to anchor orthodontic movements. Mini-screws became widely used to bear orthodontic and orthopaedic loads, becoming effective orthodontic anchorage devices [34-35]. Firstly, the material of choice to manufacture mini-implants was commercially pure Titanium (cp Ti), but in order to improve its mechanical properties, some elements such as aluminium (Al), vanadium (V) and iron (Fe) have been incorporated [36]. Many new systems were introduced, such as onplants [37], palatal implants [38], bicortical screws [39], miniplates [40], miniplates with a tube [41], and the zygoma anchorage system [42]. Although the dramatic developments and improvements of orthodontic appliances such as brackets, archwires and mini-implants are satisfying and amazing, little attention has been given to metallic ion release from orthodontic implant systems. The concern about this has been limited to orthodontic brackets and wires. To our knowledge, *in vivo* results about metallic ion release from orthodontic miniscrews have not been reported until now.

Taking into account all these facts, the aim of this study was to investigate the possible differences in metal release of aluminium (Al), copper (Cu), chromium (Cr), manganese (Mn), nickel (Ni), titanium (Ti) and vanadium (V), in oral mucosa cells of

patients subjected to conventional orthodontic appliances (brackets, archwires and bands) in comparison to patients treated additionally with mini-screws, and also with respect to a control group, by using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

2. Materials and methods

2.1. Patients and orthodontic appliances

Sixty patients between 11 and 60 years old were included in this study. The purposes of the study and the method of cell collection were explained to all subjects, and the written consent of all participants was obtained. Previously, the protocol of the study had the approval of the Ethical Committee of the University of Sevilla (Sevilla, Spain). These patients were classified into three groups. The control group (a) (8 men, 12 women) was selected between volunteers under the following inclusion criteria: nonsmokers, without oral or systemic diseases, without oral restorations, implants or prosthetics, clinically healthy oral mucosa, no previous orthodontic treatment and no occupational exposure to metals. The orthodontic group (b) (11 men and 9 women, nonsmokers) was treated with fixed orthodontic appliances in both arches.
The appliances consisted of 8 bands (Ceosa, Madrid, Spain) on the first and second permanent molars, brackets in both arches, and upper and lower 0.016 NiTi, 0.016x0.022 NiTi and/or 0.016x0.022 Stainless Steel archwires (AISI302 alloy) (Ceosa, Madrid, Spain). The orthodontic-miniscrew group (c) (10 men and 10 women, nonsmokers) required fixed orthodontic and miniscrew (Jeil Medical, Seoul, Korea). The location of the miniscrew was variable: palatal, vestibular, anterior or posterior. Each of the patients had only one miniscrew placed in mouth during sample collection.

A total of 20 self-drilling miniscrews (JA, Jeil Medical) were used, which had a cylindrical screw design of 1.6 mm in diameter and 9.0 mm in length, and had a circle-shaped head with a hole diameter of 0.9 mm. The orthodontic appliances were the same as the orthodontic group. The sample collection took place after around 15 months of orthodontic treatment (15±3 months) in groups (b) and (c).

Micro-Xray fluorescence (μXRF) measurements of the materials used in this study, including miniscrews, were performed according to Martin-Cameán et al. [24]. Results (wt.%) obtained for the composition of the miniscrew were: 6.78 Al, 88.68 Ti, 0.17 Fe, 0.13 Ni, 4.25 V

2.2. Buccal cell collection technique

The participants were asked to rinse their mouth with tepid water for 1 min to remove exfoliated dead cells. Epithelial cells of buccal mucosa from each patient were collected, using a soft rubber interdental brush without any metal content, as stated by the manufacturer (Sunstar Iberia S.L., San Just Desvern, Spain), and following the methods of Besarati Nia et al. [43] and Martin-Cameán et al. [32].

2.3. Reagents and materials

Type I water (>18MΩ·cm) obtained by a Milli-Q Element water purification system (Millipore, Bedford, MA, USA) was used throughout. All transfer pipettes, centrifuge tubes, plastic bottles, autosampler vials and laboratory ware material were made of plastic and cleaned by soaking in 20% v/v HNO_3 sub-boiling quality for 4 h, rinsing three times with type I water, according to EPA method 200.8 (United States Environment Protection Agency, version 5.4, 1994), and drying in a laminar flow hood.

Blank solution consisted of 1% v/v HNO_3, prepared by diluting 65% nitric acid

with the appropriate volume of type I water. High purity nitric acid was obtained by triple purification of 65% reagent grade nitric acid in a sub-boiling distiller purchased from Savillex (Savillex Corp., Eden Prairie, MN , USA). A tuning solution containing 10ng/mL cerium (Ce), cobalt (Co), lithium (Li), thallium (Tl) and yttrium (Y) in 1% HNO_3 was prepared from single-element (10,000 µg/mL) stock standards (AccuStandard, Inc., New Haven, CT, USA), and was used to optimize ICP-MS parameters before each analytical run. Rhodium 1 µg/mL, prepared from a 100 µg/mL stock solution (AccuStandard, Inc., New Haven, CT, USA) was used as an internal standard solution throughout the whole analysis. A standard solution containing 1 µg/mL of Al, Co, Cr, Cu, Ni and V was prepared in 100 mL Pyrex glass volumetric flask by dilution of 10 µg/mL multi-element standard solution for ICP-MS (AccuStandard, Inc., New Haven, CT, USA) with the suitable amount of blank solution. The standard solution of 1 µg/mL was subsequently diluted with the suitable amount of blank to prepare the working standards. A stock 10000 µg/mL Ti standard was purchased from AccuStandards as well and subsequently diluted with the needed amount of blank solution in order to obtain less concentrated standards.

2.4. Instrumentation and Determination of metals

All ICP-MS measurements of metal contents were carried out in an Agilent 7500c ICP-MS (Agilent Technologies, Japan). Sample introduction was performed with a PFA (perfluoroalkoxy) microflow auto-aspirating nebulizer combined with a double-pass spray chamber (Agilent Technologies, Japan). The validation of analytical procedures developed for the determination of metals in oral mucosa cells has been reported previously by our workgroup Martin-Cameán et al. [24,32], with some

modifications to improve the limits of detection (LODs) and quantification (LOQs). For this purpose, nitric acid and water metallic content were reduced, plastic ware was preferred to glassware and a more stable nebulizer was used for sample introduction. Moreover, the present work comprises the determination of most elements previously studied (Co, Cr, Cu, Ni, Ti, V) and is further expanded to include Al. Briefly, each interdental brush was introduced into a previously cleaned (4 h in 20% v/v HNO_3) 50 mL centrifuge tube, together with 10 mL of type I water and 100 µL of sub-boiling 65% HNO_3, resulting from the distillation of 65% reagent grade HNO_3 from JT Baker (Avantor Performance Materials B.V., Deventer, The Netherlands). Then, samples were heated in a water bath at 80 °C for 60 min. Afterwards, samples were cooled lightly and treated in an ultrasonic bath for 5 min. Finally, samples were cooled down to room temperature, and the acid solution was separated from the brush. Acid solutions were stored in clean 20-mL polypropylene vials at 4 °C until analysis. Less than 0.5mL were needed for the analysis.. Rhodium was used as an internal standard and was spiked at 250 ng/mL to every blank, sample and standard individually. The relative standard deviation of the concentration of Rh in all samples was calculated to be 0.85%.

2.5. Statistical analysis

Statistical analysis of the results was performed by the soft-ware Statistica (version 6.0, Statsoft, Inc., OK, USA). Descriptive statistics (means, standard deviation, medians, range and 5–95th percentiles) were reported. Normality of distribution of experimental results was assessed by the Chi-Square test. Data distribution was always found non-normal, and accordingly, non-parametric methods were applied, such as Mann–Whitney and Spearman tests. In addition, a a stepwise linear regression was

run adjusted for the presence of miniscrew along the fixed appliances, just fixed appliances or any of treatments, gender, type of braces, type of upper and lower archwires during sample collection. The data were analyzed by the Statistical Package Social Sciences (version 19) software (SPSS, Chicago, IL, USA) and *p*-values less than .05 were considered statistically significant.

3. Results

3.1. Analytical aspects

Several improvements were introduced throughout the measurements, aiming at reducing the LODs and LOQs. The *ad hoc* triple distillation of the nitric acid provides higher purity than that of available commercial solutions. Acid is distilled few hours before its intended use, and thus impurities are kept at a minimum. Same triple-distilled nitric acid was employed during the cleaning of the laboratory ware. Type I water was obtained from a Milli-Q Element source, which offers one more filter than the widespread Milli-Q sources, and reduces even further the metallic content of water. The employment of plastic labware reduces the leaching of metallic ions from the walls of the container into the sample, compared to widespread borosilicate glass, as stated by Richter (Richter, 2003) [44]. The microflow nebulizer works by auto-aspiration, taking advantage of the Venturi effect, and thus provides a more stable signal in the ICP-MS, because mechanical disturbances from the peristaltic pump are avoided.

Table 1 shows the LODs and LOQs attainable in this study, compared to those obtained previously in our lab [24,32]. All elements, except for Co, show a remarkable lowering (2-3 fold) of their LODs and LOQs, especially in the case of V.

3.2. Contents and distribution of metals in oral mucosa cells from patients.

The cellular content of the eight elements were measured for all the patients, and in the case of cobalt and vanadium, only traces were detected in a small number of samples (1-2%), being their contents lower than their respective LODs. Then, these two elements will be not further considered. The concentration of Al, Cr, Cu, Ni and Ti levels in oral mucosa cells were fitted to typical statistical distributions (Gaussian, log normal, gamma) and are shown in Figure 1. None of the elements were normal distributed, and all further statistical computations were based on non-parametric techniques. Table 2 shows the descriptive statistics for the elements in the three groups of patients considered (control, orthodontic and orthodontic+miniscrew). In order to make comparisons with the scientific literature mean values (± sd) are provided. Metal release values in oral mucosa cells was variable in all the patients considered, mainly in the case of Cu. The lowest levels were found for Cr whose medians were 0.18 ng/mL in control and orthodontic group, and 0.23 ng/mL in orthodontic+miniscrew group. Ni values in control group (median=0.26 ng/mL) were significantly lower in comparison to orthodontic patients (0.48 ng/mL) ($p<0.05$) and to orthodontic+ miniscrew group (0.45 ng/mL) ($p<0.05$). Similar levels were found for Ti, whose medians were 0.38, 0.28 and 0.35 ng/mL in control, orthodontic and orthodontic+miniscrew groups, respectively. In the case of copper levels, a significant increase was found in orthodontic group (median=0.89 ng/mL) when compared to control group (0.57 ng/mL) ($p<0.05$). Aluminium was the predominant element with medians about 12 ng/mL for all the patients considered.

3.3. Interrelations between elements

To statistically prove possible correlations between the contents of the studied elements in oral mucosa cells the non-parametric technique of Spearman [45] was employed. The only correlation (with a probability (P) of error level less than 0.05 was Al/Ti (r = 0.469), and this correlation was positive, that is, both levels change in the same direction.

Following the procedure described by Martin-Cameán et al. [26] and despite of the non-normality of the distributions of metals studied in oral mucosa cells, the element-to-element linear regression plots for statistically significant correlations were evaluated. The analysis of correlation coefficients shows that there is significantly positive correlations ($p<0.05$) only in the case of Al/Ti ($r=0.36$) (Figure 2).

3.4. Multiple linear regression analysis of the influence of orthodontic treatment variables on the ion concentrations

The levels of metallic elements can be influenced by different factors such as gender, type of treatment including type of braces and archwires and also it might be influenced not simply by the level of another element, but by the level of the group of elements potentially released from orthodontic appliances and miniscrew. In this regard, the relationships among different elements and clinical factors related to the treatment, and between their concentrations were examined by a stepwise linear multiple regression analysis [46] adjusted for variables described above (Table 3). Results from the regressive analysis give no relevant information regarding a linear correlation or influence of any of the included variables in the matrix with any of the metallic ions (p >.05). Therefore as compiled in Table 3, none of the clinical variables are useful predictors of the results of the present study (p > .05)

4. Discussion

In vivo metal release from orthodontic appliances has been reported using different techniques and experimental procedures, and a systematic review of this matter has been published by Mikulewicz and Chojnacka [15]. These reports deal with traditional orthodontic appliances, but miniscrews are also a potential source of metals not evaluated before.

The biological samples usually investigated have been saliva [4, 6-7,12,47-50], there were also some works relative to serum, urine, and more recently reports in hair [26, 51-52] and mucosa oral cells [4, 9-10, 12,17], whose advantages of these latter matrices have been reported [24]. Regarding the ICP-MS technique employed to determine metals from these samples, the procedure for sample pretreatment and determination of metals in oral mucosa cells have been validated in our laboratory [24, 32]. In this work, the adopted strategy carried out to reduce the background due to metallic contribution from reagents and instrumental instabilities, have had a positive impact on LOD and LOQs values of the elements analysed by ICP-MS.

The release of the elements studies showed the following ascending order: Cr < Ni < Ti < Cu < Al, and two metals, V and Co were considered as not detected in the samples analysed. Vanadium has been not previously determined in oral mucosa cells [4, 9-10, 12, 17] or was not detected [24]. However, although V is present in some materials (bands 0.18-0.19%, and miniscrew 4.25%), the application of miniscrew did not contribute to a significant release of this metal. Cobalt was only present in the stainless steel arch (0.62%) and its null release is in agreement with Fernandez-Miñano et al. [17] who evaluated three alloys in brackets and tubes: stainless steel, titanium and nickel- free. Also, Amini et al. [12] did not find differences in the concentration of

Co of patients treated with conventional fixed appliances after 16 months. However, Faccioni et al. [10] found a significant 2.8-fold increase in the concentration of Co released during the 24-48 months period of treatment. In this case, some of the archwires used were chromium-cobalt-nickel alloy and a considerable percentage of Co (42%) was included in its composition.

In general, among stainless steel and nickel –titanium corrosion products, Ni and Cr have received the most attention because of their reported adverse effects [26]. In relation to Ni, the increased release observed in all experimental groups with respect the control in the present study agreed with those reported by Faccioni et al. [10] who found 3.4-fold higher Ni values in orthodontic patients than those in the control subjects. This finding could be explained because Ni is a common constituent of the standard orthodontic appliances, especially in Ni-Ti arches. Similarly, Amini et al. [12] confirmed that nickel contents in mucosa cells of orthodontic patients were significantly higher (2-fold) f than in controls. Moreover Hafez et al. [4] studied Ni release with the time in a longitudinal controlled clinical trial, and reported an increase of Ni cellular content from 0.52 to 0.68 and 0.78 ng/mL in buccal mucosa cells, after 0, 3 and 6 months of treatment, respectively. However, Natarajan et al. [9] did not find significant differences in Ni ion contents after 30 days of debonding. Therefore, the study period is a factor to take into account. Nickel is well-known for producing contact dermatitis in sensitized persons that can become quite severe [53], and adverse reactions related to Ni-containing orthodontic devices (archwires, brackets, soldered stainless steel face-bows) have been reported [54]. Very low concentrations of Ni^{2*} could induce gene activation in endothelial cells, similar to pro-inflammatory

mediators like interleukin6 (IL-6) and interleukin 8 (IL-8) and provoke inflammatory reactions and may modulate the immune response by activation T-and B-cells [8]. This element has carcinogenic and mutagenic effects [55], and consequently, the exposure to it should be as low as reasonably possible.

Regarding titanium levels, due to its mechanical properties, good resistance to corrosion in biological fluids and very low toxicity, this metal has been the most commonly selected material for dental implants and prostheses [56-57]. Despite its common presence in orthodontic fixed appliances (NiTi archwires), including miniscrews, no increased release was detected in this work. Other authors however, showed enhanced values of Ti in oral mucosa cells [17]. This element has not been investigated in saliva.

Data relative to Cr release in oral mucosa cells are contradictory. Some reports indicated higher contents of this metal in oral mucosa cells in comparison to control groups [4,17], whereas our results agreed with Amini et al. [12] and Natarajan et al. [9] who did not find an increase of the release of this metal. In other matrices, such as saliva, the release of Cr and Ni from orthodontic appliances was assessed by Amini et al. [12] and these authors did not find significant release of Cr, but higher levels in the case of Ni in 28 subjects who had undergone fixed orthodontic therapy for 12-18 months. Moreover, most of the experiments measured ion release during the exposure to orthodontic appliances to a biological medium (blood, serum, urine, or saliva) for periods ranging from 1 day to 1 month or even over a 10-month period. These reports have been recently revised by Matusiewicz [58], and in general, contradictory results have been found in salivary metal ion concentrations between subjects with and without fixed orthodontic appliances.

In relation to copper contents, the only significant increase observed in the orthodontic group and not in the orthodontic+miniscrew group could be due to the high variability of the results. Release of Cu from orthodontic appliances has been not extensively studied in the scientific literature, although it is commonly present in many alloys [59] used in the orthodontic practice. Recently, Mikulewicz et al. [27] studied *in vivo* the Cu release (and other elements) in non-invasive (hair) and invasive matrices in pigs who received plates simulating the orthodontic appliance, and found significant differences in the levels of this element in liver, kidney, aorta, lung and hair of experimental pigs.

Aluminium is the third most common element (about 8%) of the earth's crust, and it is an extremely versatile metal with a wide variety of uses, and is non-essential for humans, resulting in low-level distribution in human organs [60, 61]. Its ubiquity could explain the highest levels observed in all the patients. However, its oral bioavailability in humans and experimental animals is low; from drinking water it has been estimated to be in the range of 0.3%, whereas from food and beverages generally is considered to be lower, about 0.1% [62]. In relation to orthodontic appliances composition, aluminium is present in the miniscrew, so this is the first time that its potential release has been evaluated. According to the results obtained, no differences were found in the three experimental groups studied. Therefore, miniscrew resulted biocompatible with respect to this metal.

In the case of orthodontic patients, the studies on inter-element interactions are scarce and only limited to levels contained in hair. Synergism has been reported between Fe and Cr by Mikulewicz et al. [51], and also positive correlations between Cu/Mn, Cu/Ni and Fe/Ni were found in a previous report from our laboratory [26].

These interactions could be explained in part by the common origin of elements, the miniscrew, or synergistic effects between them.

Linear correlation and presence of potential confounding clinical factors affecting the observed results were explored by a regression analysis of these clinical variables and the metals concentrations. No evidence was found of any statistical correlation of gender, presence of miniscrews, type of braces neither archwires in the lower and upper jaws and between metallic ions. These results might be partially explained due to inherent limitation in sample size for finding linear associations. However, interestingly, these results imply that miniscrews are safe auxiliary orthodontic devices in term of metallic ion releasing to the mouth as previous studies have concluded *in vitro* [63] and in the animal model [64]. Nevertheless, no data are found to date in this or other studies regarding the ionic releasing into the alveolar bone since those mini-implants are directly screw into the alveolar bone without any surgical approach; and screwing friction is a well-known factor that induces severe damage in the metallic surface [65]. These potential effects might be of substantial interest for future studies in the field.

Finally, no adverse effects were observed in any patient treated in our study, and it would be explained because the quantified amounts of the elements released are below their respective Tolerable daily intake (TDI) in the case of Cu (5 mg/day [66] , Ni (12 µg/Kg body weight) [67], or Tolerable weekly intake (TWI) for Al of 1 mg/Kg boy weight/week for its cumulative nature in the organism [62], and with the Tolerable Upper Intake Level (UL) for Cr (1 mg/day) [68]. Neither Joint of Expert Committee on Food Additives (JECFA) nor the World Health Organization (WHO) have determined

relevant tolerable intake for titanium against to compare the values of metal ions released obtained [69].

Conclusions

The modifications introduced in the pretreatment of oral mucosa cells reduce the background due to metallic contribution from reagents and instrumental instabilities, and have a positive impact for an adequate *in vivo* determination by ICP-MS of Al, Cu, Cr, Mn, Ni, Ti and V released in patients with fixed orthodontic appliances. Only traces of Co and V were detected, whereas the release of the elements studied in patients showed the following ascending order: Cr < Ni < Ti < Cu < Al. Differences in the content of metals in oral mucosa cells were only significantly increased for Ni in orthodontic and ortohodontic+miniscrew groups, and also in Cu levels in orthodontic group, in comparison to the control group. Nevertheless no linear correlation resulted for none of the ions with regard to the type of orthodontic fixed appliance used, with or without miniscrew. In general, the incorporation of miniscrews assayed did not imply a significant increase of metal release. A positive correlation between Al/Ti was found, probably due in part to their common origin, or synergistic effects between them.

Acknowledgements:

This work was supported by the Junta de Andalucía (Plan Andaluz Investigación, code CTS-358).

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure captions

Figure 1. Distributions of metal concentrations determined in oral mucosa cells.

Figure 2. Interrelation between Al/Ti determined in oral mucosa cells.

Table captions

Table 1. Comparison of LODs and LOQs of the present study with previous works

Table 2. Metal levels in oral mucosa cells from the control group (n = 20), orthodontic group (n=20) and orthodontic+miniscrew group (n=20) studied.

Table 3. Forward stepwise multiple linear regression analysis of metal ion concentration detected in the patient´s mucosa cells.

*Conflict of Interest Statement

AUTHOR DECLARATION TEMPLATE

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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CAPÍTULO 5 / CHAPTER 5

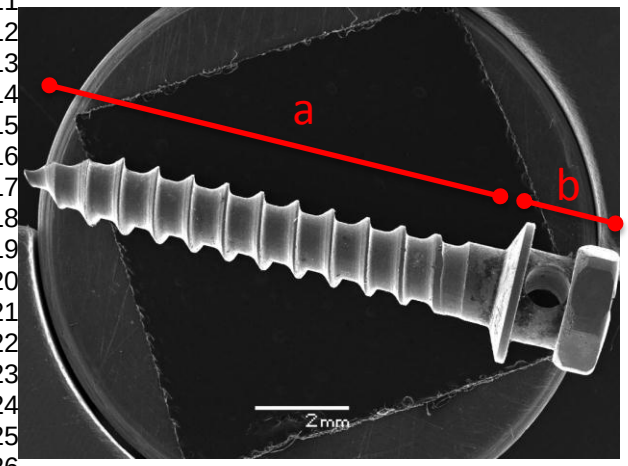
Ana Martín-Cameán, María Puerto, Ángeles Jos, Amaya Azqueta, Alejandro Iglesias-Linares, Enrique Solano, Ana M. Cameán

EVALUATION OF GENOTOXICITY OF ORTHODONTIC MINISCREWS ON MUCOSA ORAL CELLS BY THE ALKALINE COMET ASSAY

Toxicology Mechanisms and Methods (en revisión). **2015**

Evaluation of genotoxicity of orthodontic miniscrews on mucosa oral cells by the alkaline comet assay

Journal:	*Toxicology Mechanisms and Methods*
Manuscript ID:	Draft
Manuscript Type:	Original Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Martin-Cameán, Ana; University of Sevilla, Puerto, Maria; University of Sevilla, JOS, ANGELES; University of Sevilla, Azqueta, Amaya; Universty of Pamplona, Iglesias-Linares, Alejandro; University Complutense, Solano, Enrique; University of Sevilla, Camean, Ana; University of Sevilla,
Keywords:	Miniscrew, genotoxicity, comet assay, buccal cells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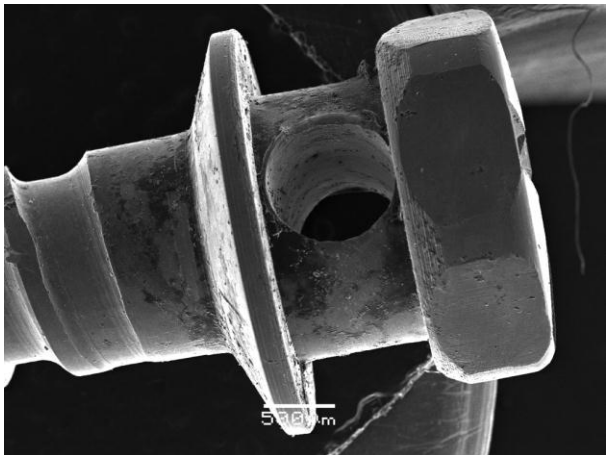

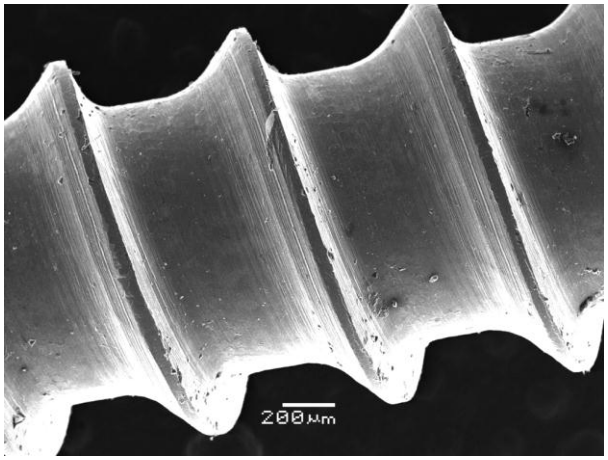
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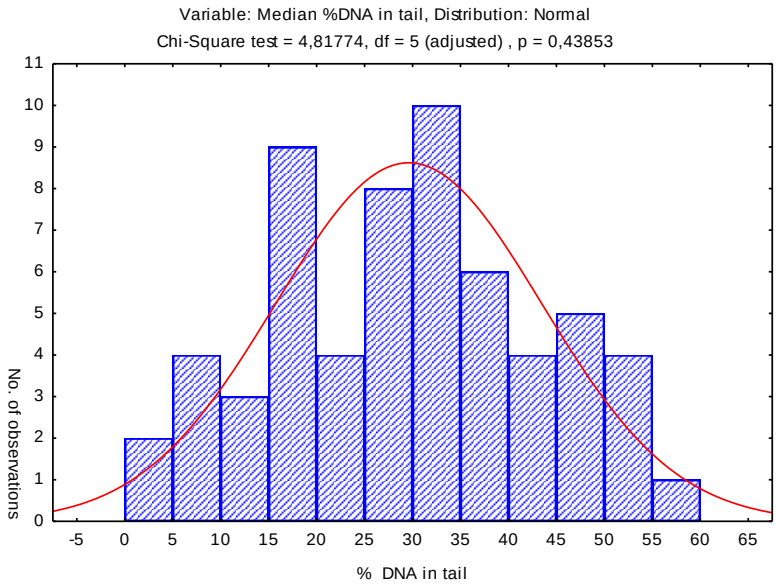
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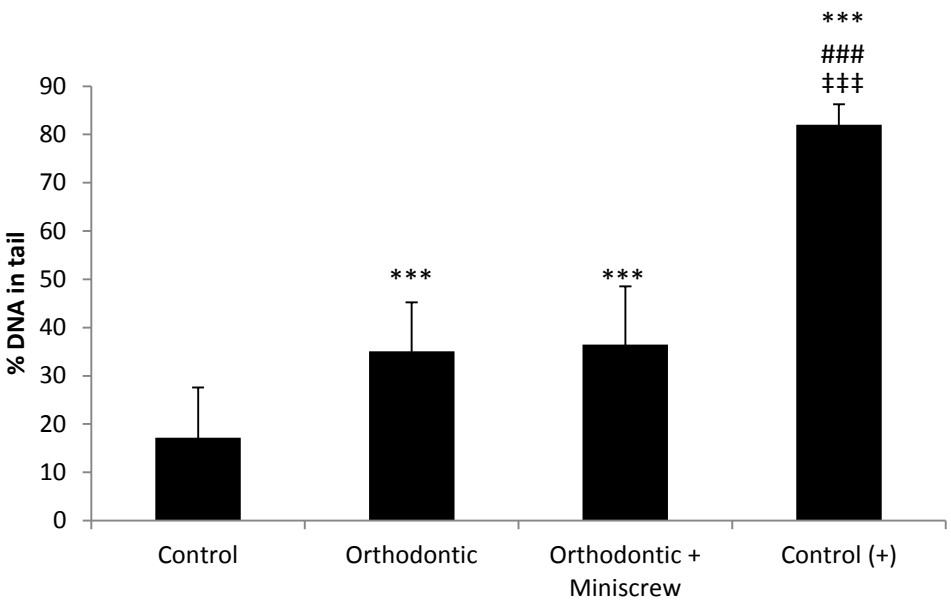
No. of observations
% DNA in tail


***
###
‡‡‡
90
80
70
60
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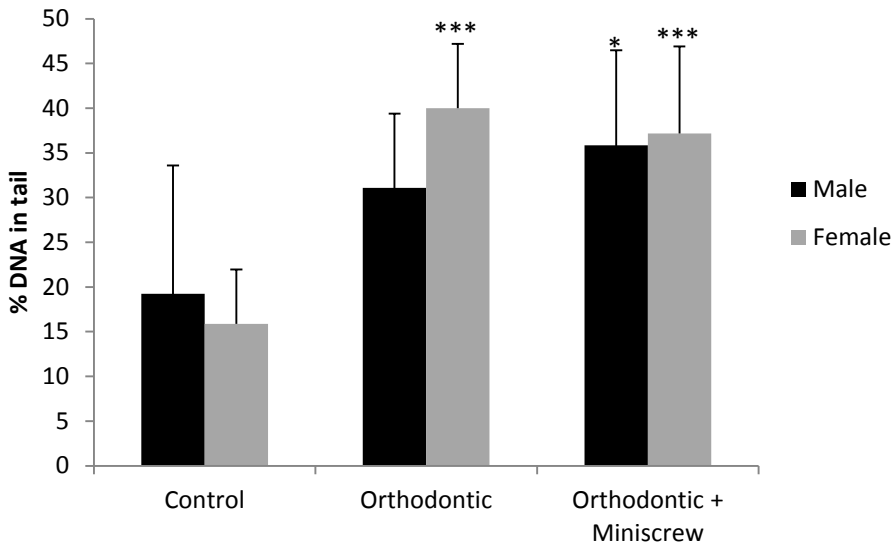
40
30
20
10
0
% DNA in tail
***
***
Control
Orthodontic
Orthodontic + Miniscrew
Control (+)


% DNA in tail
50
45
40
35
30
25
20
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5
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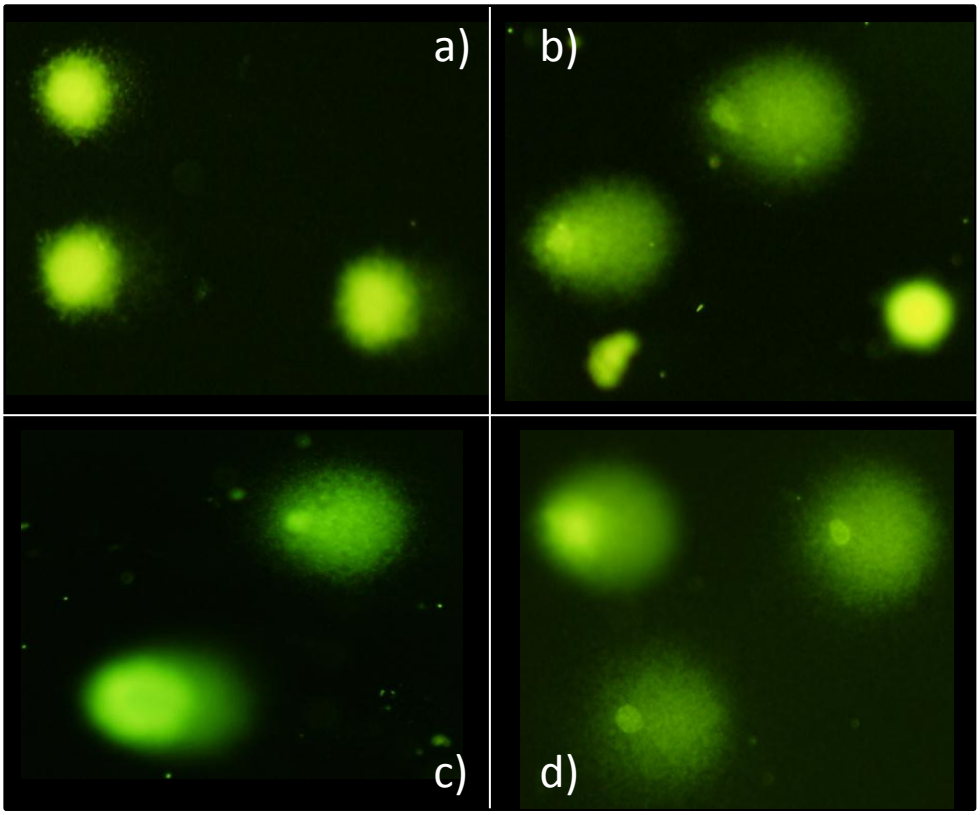
*
***
Control
Orthodontic
Orthodontic + Miniscrew
Male
Female


a)
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d)

Table 1. Chemical composition of the orthodontic appliances and miniscrew.

Material - Product	Composition (wt.%)
Stainless steel - Ligature .010	18.93 Cr, 0.50 Cu, 70.37 Fe, 0.39 Mo, 9.58 Ni, 0.23 Rb
Stainless steel - Ligature .012	18.77 Cr, 0.30 Cu, 70.57 Fe, 0.21 Mo, 9.94 Ni, 0.20 Rb
Band single tube	17.51 Cr, 0.60 Cu, 69.59 Fe, 2.06 Mo, 9.75 Ni, 0.29 Rb, 0,19 V
Band double tube	18.66 Cr, 0.31 Cu, 68.80 Fe, 2.22 Mo, 9.63 Ni, 0.19 Rb, 0.18 V
Bracket BioMesh	18.42 Cr, 0.37 Cu, 66.94 Fe, 2.47 Mo, 11.57 Ni, 0.23 Rb
0.016 Nickel-Titanium arch	0.12 Cr, 56.33 Ni, 43.42 Ti, 0.13 Zr
0.016x0.022 Nickel-Titanium arch	0.10 Cr, 55.9 Ni, 43.88 Ti, 0.12 Zr
0.016x0.022 Stainless Steel arch (AISI302 alloy)	18.74 Cr, 0.61 Cu, 72.38 Fe, 0.24 Mo, 7.41 Ni, 0.62 Co.
Miniscrew	6.78 Al, 88.68 Ti, 0.17 Fe, 0.13 Ni, 4.25 V

Evaluation of genotoxicity of orthodontic miniscrews on mucosa oral cells by the alkaline comet assay

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Keywords: Miniscrew, genotoxicity, comet assay, buccal cells

Abstract

Miniscrew implants are widely used nowadays in orthodontic treatments due to their good results in clinical practice. However, data regarding the biocompatibility of commercially available orthodontic miniscrews and temporary devices are very scarce, and their role as genotoxicity inducers has been not previously evaluated with the alkaline comet assay. The aim of this study was to investigate the DNA damage in buccal cells of patients subjected to orthodontic treatments. The alkaline comet assay has been applied in oral mucosa cells from patients treated with conventional orthodontic treatment in comparison to patients treated additionally with miniscrews, non-treated volunteers (control) and smoking volunteers (positive control). The application of orthodontic appliances and miniscrews induced significant and similar (2-fold) increases of % DNA in tail in comparison to control group. Females experienced a significant increase in %DNA in all the treatments in comparison to the control group, whereas males showed significant damage only with the combined orthodontic and miniscrew treatment. In conclusion, conventional orthodontic appliances induced genotoxicity, and the incorporation of miniscrews assayed did not imply any additional increase of DNA damage.

1. Introduction

Orthodontic miniscrews have been extensively popularized worldwide because of their simplicity of management, low cost and the minimal need for patient compliance (Papadopoulos and Tarawneh, 2007; Papageorgiou et al., 2012). The value of miniscrew implants is in the prerequisite that they remain relatively stationary in the bone, their ability to increase anchorage capacity, and the absence of adverse effects or complications that could endanger health or treatment outcome (Liou et al., 2004; Papageorgiou et al., 2012).

Although the developments and improvements of orthodontic temporary anchorage devices (TADs) are satisfying, their biocompatibility is an important question that remains to be fully elucidated not only in the animal model but also in humans (Malkoc et al., 2012). Despite the high increase in the use of these type of devices in orthodontics in recent years, data in the orthodontic literature regarding the biocompatibility of commercially available orthodontic TADs are still very scarce (Morais et al., 2007; De Morais et al., 2009; Malkoc et al., 2012).

In the oral cavity, many factors work together to create an environment that makes aqueous corrosion in metals and alloys more favourable (Burrows, 1986; Natarajan et al., 2011). Saliva acts as an electrolyte for electron and ion conduction, and the fluctuation of pH and temperature, the enzymatic and microbial activity, and the various chemicals introduced into the oral cavity through food and drink are all corrosion conductors (Eliades and Bourauel, 2005; Hafez et al., 2011). The released metals can induce adverse biologic effects, such as cytotoxicity, and they are suspected genotoxic agents (Faccioni et al., 2003). The genotoxic properties of

metals from orthodontic appliances are defined as an essential criterion to select these materials in a safe biological manner for patients (Montanaro et al., 2005; Westphalen et al., 2008). Genotoxicity tests can be defined as *in vitro* and *in vivo* approaches designed to detect compounds that induce genetic damage, including DNA lesions, gene mutation, chromosomal breakage, altered DNA repair capacity, and cellular transformation (Angelieri et al., 2011). In the case of orthodontic appliances, biocompatibility data from *in vivo* human studies are needed in order to evaluate all potential risks (Angelieri et al., 2011). Indeed, the limited data existing on the biocompatibility of them appear to be insufficient (de Morais et al., 2009), and has been mainly limited to orthodontic brackets and wires (Faccioni et al., 2003; Westphalen et al., 2008; Angelieri et al., 2011; Fernández-Miñano et al., 2011; Hafez et al., 2011; Natarajan et al., 2011; Ortiz et al., 2011).

The assessment of genotoxic agents can be performed through the analysis of primary DNA damage, as accessed by the comet assay or alkaline single cell gel electrophoresis (Westphalen et al., 2008). It measures single- and double-strand breaks and alkali-labile sites in each individual cell (Singh et al., 1988). The utility of the comet assay lies in its requirement for very small numbers of cells and on its ability to evaluate DNA damage in either proliferating or non-proliferating cells (Hartmann and Speit, 1994). The comet assay is considered a quick, simple, sensitive, reliable, and fairly inexpensive way of measuring DNA damage (Collins et al., 2002). The use of buccal cells in the assay was reported for the first time for biomonitoring studies of DNA damage of smokers (Rojas et al., 1996), and also to investigate differences in the basal level of DNA damage between young adults exposed to air pollution in different areas of Mexico city (Valverde et al., 1997). These authors reported the feasibility of using the comet assay in the monitoring of

humans potentially exposed to genotoxic pollutants (Valverde et al., 1997). The published protocols used in these reports of buccal cells, collected easily from the inside of the mouth, showed sustained massive damage and disintegration at the high pH used. They were improved by Szeto et al., (2005), demonstrating that their buccal cell comet assay was a feasible and potentially useful alternative tool to the usual lymphocyte model in human biomonitoring and nutritional work.

The comet assay is the method of choice for measuring DNA damage in human cells obtained in the course of population-based studies of exposure to different to genotoxic agents (Dusinska and Collins, 2008). But, to our knowledge, investigations into the in vivo genotoxic potential of miniscrews in mucosa oral cells from humans using this methodology have not been reported previously. Taking into account all these facts, the purpose of this study was to investigate inter-individual differences in buccal cell DNA damage (as strand breaks and alkali labile sites) in patients subjected to conventional orthodontic treatment (brackets, archwires and bands) in comparison to patients treated additionally with miniscrews. In addition an external control group of volunteers was used to compare analyzed data.

2. Materials and Methods

2.1. Patients and type of orthodontic appliances.

Seventy patients between 11 and 60 years old were included in this study. The purposes of the study and the method of cell collection were explained to all subjects, and the written consent of all participants was obtained, according to Hafez et al., (2011). Previously, the protocol of the study had the approval of the Ethical Committee of the University of Sevilla (Sevilla, Spain) in compliance with the World Medical Association Declaration of Helsinki. These patients were classified into four

groups. The control group (a) (8 men, 12 women) was selected from volunteers under the following inclusion criteria: nonsmokers, without oral or systemic diseases, without oral restorations, implants or prosthetics, clinically healthy oral mucosa, no previous orthodontic treatment and no occupational exposure to metals (Hafez et al., 2011). The orthodontic group (b) (non-smokers:11 men, 9 women) was treated with fixed orthodontic appliances in both arches. The appliances consisted of 8 bands (Ceosa, Madrid, Spain) on the first and second permanent molars, brackets in both arches, and upper and lower 0.016 NiTi, 0.016x0.022 NiTi and/or 0.016x0.022 Stainless Steel archwires (AISI302 alloy) (Ceosa, Madrid, Spain) (Martin-Cameán et al., 2014). The orthodontic-miniscrew group (c) (non-smokers:10 men, 10 women) required both fixed orthodontic and miniscrew appliances (Jeil Medical, Seoul, Korea). The location of the miniscrew was variable: palatal, vestibular, anterior or posterior. Each patient had only one miniscrew in the mouth at the time of sample collection. A total of 20 self-drilling miniscrews (JA, Jeil Medical) were used, which had a cylindrical screw design of 1.6 mm in diameter and 9.0 mm in length, and had a circled-shaped head with a hole diameter of 0.9 mm. The orthodontic appliances in this group (orthodontic+miniscrew) were the same as the orthodontic group. The sample collection took place after around 15 months of orthodontic treatment (15±3 months) in groups (b) and (c). A fourth group (d) composed of 10 smoking volunteers (5 men, 5 women) was included as positive control, as they are known to suffer higher DNA damage in exfoliated buccal cells (Rojas et al., 1996).

The microstructure of the orthodontic miniscrew was analyzed using scanning electron microscopy with a Jeol 6460 LV microscope (JEOL USA, Inc.) operated at 20 kV. The images obtained are shown in Figure 1. Micro-Xray fluorescence (μXRF) measurements of the materials used in this study, including miniscrews, were

performed in an EAGLE III [energy-dispersive analysis by X-rays (EDAX)] energy dispersive micro-X-ray fluorescence spectrometer equipped with a Rh X-ray tube, 300-µm monocapillary optics, a charge-coupled device (CCD) camera, and an 80-mm2 Si (Li) detector (Alba et al., 2010; Martin-Cameán et al., 2014). The composition of these materials is shown in Table I.

2.2. Buccal cell sampling

A cytobrush was used for sampling, which appears to be the most effective technique for collecting large numbers of oral mucosa cells (Holland et al., 2008). First a soft bristle toothbrush was used to remove the first layer of the buccal mucosa cells by scraping the inside cheek of the mouth gently after rinsing the mouth with distilled water (Szeto et al., 2005). This sample was discarded and a second toothbrush was used to collect buccal cells and treated following Szeto et al., (2005). All samples were collected and rapidly processed.

2.3. Comet assay

The method used herein is similar to the reported by Collins et al. (1997), Szeto et al. (2005), Hafez et al. (2011) and Collins and Azqueta (2012) but with some modifications. In order to obtain nucleoids from oral mucosa cells the lysis solution included 0.1 % DMSO and a subsequent digestion with 10 mg/ml proteinase K for up to 1.5 h in a metal-box at 37 ºC was performed. The medium-throughput format of 12 minigels per slide (two rows of six minigels in a microscope glass slide) was used (Shapashnikov et al., 2010; Azqueta et al., 2013) and 3 samples from the same patient were analyzed on each slide. Briefly, cell suspension were mixed with agarose and placed on agarose precoated slides. After the lysis of the cells (4 ºC),

their digestion with proteinase K and the denaturation of the DNA in an alkaline buffer 20 min (4 ºC), the electrophoresis was carried out at approximately 1 V/cm for 20 min (4 ºC). Finally, once the slides were washed and air dried, nucleoids were stained with SYBR Gold and visualized according to the conditions reported by us earlier (Maisanaba et al., 2013b). Images of at least 100 randomly selected nuclei per patient were analyzed. Percentages of tail DNA, automatically obtained by the software, were used to describe each of the nucleoids/comets analyzed and the median of the scored comets was obtained to describe each patient/volunteer.

2.4. Statistical analysis

Mean ± SD of the medians were calculated for each group of patients (groups b,c) or volunteers (groups a,d). Statistical analysis of the results was performed by the soft-ware Statistica (version 6.0, Statsoft, Inc., USA) and GraphPad InStat3 software (La Jolla, CA, USA). Normality of distribution of experimental results was assessed by Chi-Square test (Mikulewicz et al., 2011). Data distribution was always found normal, and accordingly, parametric methods were applied (Martin-Cameán et al., 2014). The percentages of DNA in tail were assessed by analysis of variance (ANOVA) followed by TuKey-Kramer multiple comparison tests, contrasting control and exposed groups, and male versus female. Differences were considered significant from $p < 0.05$.

3. Results

The potential genotoxic effects of fixed orthodontic appliances, and specifically miniscrews were evaluated by the alkaline comet assay. The distribution of %DNA in

tail in human mucosa oral cells was fitted to a typical normal statistical distribution (Figure 2). The application of orthodontic appliances and miniscrews induced significant and similar (2-fold) increases of % DNA in tail in comparison to control group ($p<0.001$), and no statistical differences between the two orthodontic treatments were found (Figure 3). Moreover, the positive control group (smokers) experienced significant increases of % DNA in tail (>4-fold) with respect to the control group of patients and to the experimental groups of patients submitted to orthodontic treatments (≈ 2-fold).

We have considered the possible influence of sex, and data for the bivariate analysis among genotoxic biomarkers and sex are presented in Figure 4. In general, females belonging to orthodontic group (n=9) and orthodontic+miniscrew group (n=10) had significant increases in % DNA in tail (2.5-fold, $p<0.001$; 2.3, $p<0.001$) with respect to the control group (n=12). Males experienced a slight non-significant increase of % DNA in tail in the orthodontic group whereas a 2-fold significant increase was observed in the orthodontic + miniscrew group (n=10) in comparison to the control group (n=8)($p<0.05$). There were no significant differences related to the sex for any of the treatments.

Images of cells from the different experimental groups, with undetectable DNA damage (control group) (a), and typical comets in orthodontic (b), orthodontic+miniscrew (c), and positive control group (smokers, d) are shown in Figure 5.

4. Discussion

Nowadays, there are four main areas of research in which the comet assay has been adopted: *in vitro* and *in vivo* genotoxicity testing, human biomonitoring,

ecogenotoxicology and basic research into mechanisms of DNA damage and DNA repair (Collins, 2004; Azqueta et al., 2011; Azqueta and Collins, 2013). The comet assay is a widely used biomonitoring tool for DNA damage and the buccal cell model constitutes an attractive and potentially useful tool for investigating *in vivo* effects on DNA damage of dietary agents, lifestyle choices, chemical agents and xenobiotics in general (Szeto et al., 2005; Pereira da Silva et al., 2012; Collins et al., 2014). The genotoxicity of orthodontic appliances is of serious concern. Persistent DNA damage can lead to mutations and cancer (Hafez et al., 2011). In a labile tissue such as the buccal mucosa, cellular proliferation of a damaged cell might produce many defective cells (Hafez et al., 2011).

Material biocompatibility tested by in vitro methods lacks the simulation of the oral cavity with its multifactorial environment (Hafez et al., 2011). Moreover, current *in vivo* human studies are aimed at representing the real condition of the oral cavity by sampling buccal cells, which are directly exposed to the appliances (Faccioni et al., 2003; Westphalen et al., 2008). Besides, there are different reasons that support the use of this cell type, among others: it is the least invasive method available for measuring DNA damage, and these cells could represent a preferred target site for early genotoxic events induced by carcinogenic agents (Holland et al., 2008; Westphalen et al., 2008). In this regard, orthodontic appliances have been shown to induce genotoxicity in oral mucosa cells by using the alkaline version of the comet assay, although the number of *in vivo* human studies on this topic is still scarce (Faccioni et al., 2003; Westphalen et al., 2008; Fernández-Miñano et al., 2011; Hafez et al., 2011). These studies deal with traditional fixed orthodontic appliances including brackets, bands, archwires, but as far as we know this is the first study that evaluated the possible influence of miniscrews in the genotoxic response.

In all these works the buccal cells were harvested by gentle scraping of the surface of the cheek using only one soft toothbrush. In this study, we have compiled these methods including some modifications. Thus, our first soft toothbrush was used to remove the first layer of the buccal mucosa and exfoliated dead cells, and the second toothbrush was used to collect the buccal cells. In this way, we assured a better performance of the assay as dying cells do not interfere. Although exfoliated buccal mucosa cells were used previously to evaluate DNA damage, the buccal cells used in this study come from the prickle cell layer (or stratum spinosum), that could show less genotoxic effect than other layers more exposed to toxic agents (Holland et al., 2008).

Regarding to methodological aspects of the comet assay, Östling and Johanson (Ostling and Johanson, 1984) first reported the use of lysis and electrophoresis at pH 9.5. Later, Szeto et al. (2005) developed a neutral comet assay (electrophoresis at pH 9) for buccal cells including a previous proteinase K treatment. These conditions gave an acceptable comet image as reported by the authors. Singh et al. (Singh et al., 1988) modified the method by increasing the pH of electrophoresis to >13 (with 0.3 M NaOH). Also, Hafez et al. (2011) evaluated DNA damage of buccal cells including an additional step with proteinase K for 45 mn to enhance the lysis step as recommended by Szeto et al. (2005), and these conditions stretched the supercoiling of the DNA strands and produced migration in approximately 25% of the control cells. In the present work the addition of proteinase K (10mg/ml) treatment produced complete lysis of buccal cells. Moreover, we have used the alkaline version of the comet assay (pH >13 to unwind the DNA and during the electrophoresis) – the most widely used method today (Collins, 2004; Collins and Azqueta, 2012). All these modifications improve the quality of the nuclei obtained,

and result in about 20% DNA in tail in the control group, in comparison to approximately 25% obtained by other authors (Fernández-Miñano et al., 2011; Hafez et al., 2011).

In this research, the comet assay results on human buccal cells revealed that conventional orthodontic appliances (20 orthodontic patients) and the additional employment of miniscrews (20 patients with orthodontic+miniscrew) induced genetic damage in comparison to a control group (20 control subjects), although the genotoxic effects of orthodontic miniscrews were similar to patients wearing traditional fixed orthodontic appliances. These results agree with previous research by Faccioni et al., (2003) after 2-4 years of conventional orthodontic treatment (55 orthodontic patients and 30 control subjects). By contrast, Westphalen et al. (2008) found that after 10 days of the placement of the orthodontic appliances, the comet assay results indicated that these appliances did not induce any genetic damage. Similar findings were observed when the comet assay was carried out in cultured human gingival keratinocytes exposed for up to 14 days to orthodontic brackets (Tomakidi et al., 2000). These differences may be explained by the samples analyzed, larger number of patients using the appliances for a much longer period (1.5-3 years), and composition of orthodontic appliances.

According to Fernández-Miñano et al. (2011) the genotoxicity induced in buccal cells could be related with the composition of the orthodontic appliances employed. However, they considered that the orthodontic apparatus made with titanium were not toxic for the oral mucosa cells. By contrast, Hafez et al. (2011) in a longitudinal *in vivo* study observed that stainless steel brackets with stainless steel archwires showed the least biological damage, whereas the titanium brackets with nickel-titanium archwires produced the greatest cytotoxicity and genotoxicity (using

the comet assay). They also reported damage to the DNA in mucosa cells at 3 months of treatment and not at 6 months, and this might imply recovery from the initial insult and tolerance or DNA repair. Thus, regarding the time of treatment results are contradictory, with authors indicating a recovery and others higher effects.

In this work, from a general point of view, we have not found additional DNA damage in patients with orthodontic treatment+miniscrew, despite this being a more invasive procedure, and in closer contact with the buccal cells. Taking into account that the major component of miniscrews is Ti (followed by Al and V), this could not be the main responsible of the genotoxicity observed. When the sex is considered, females follow the same trend. Men also showed an increase in the genotoxic damage but this was only significant in the %DNA in tail of the orthodontic+miniscrew group. No significant differences were found between males and females in all groups of patients assayed. No data in the scientific literature have been found relating the influence of sex on the genotoxicity of orthodontic appliances, but from a general point of view Slyskova et al. (2014) reported that sex is a factor significantly associated with the level of DNA damage and that this damage is higher in women. We would like to indicate that this is a preliminary study in which a reduced number of patients has been considered. Therefore, Dusinska and Collins (2008) indicated that one of the main advantages of the comet assay was that it requires far smaller number of subjects and much less time than conventional epidemiology assays. Nevertheless, confirmation of the differences observed in the genotoxic potential between males and females in orthodontic and orthodontic+miniscrews groups is necessary. The little research in this field, the importance of this subject for the health status of orthodontic patients and the wide use of miniscrews for distal or mesial movement of teeth, anterior retraction or protraction of teeth, intrusion or extrusion,

etc., are reasons for further studies with the use of miniscrews with different compositions, larger samples, and long-term follow-up analysis.

5. Conclusion

The modifications introduced in the comet assay are satisfactory to evaluate *in vivo* the potential genotoxicity induced by fixed orthodontic appliances in oral mucosal cells. On the basis of the results obtained, we can say that the conventional orthodontic appliances induced genotoxicity, and the incorporation of miniscrews assayed did not imply a significant increase of DNA damage when all the patients are considered. In relation to sex, no differences were found between males and females in all groups of patients considered. Females experienced a significant increase in %DNA in all the treatments in comparison to the control group, whereas males showed significant damage only with the combined orthodontic and miniscrew treatment. To confirm these results further *in vivo* studies are required.

Acknowledgements:

This work was supported by the Junta de Andalucía (Plan Andaluz Investigación, code CTS-358).

Declaration of interest

The authors report no declarations of interest

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1966.

Table captions

Table I. Chemical composition of the orthodontic appliances and miniscrew.

Figure captions

Fig. 1. Scanning electron microscope energy-dispersive x-ray spectroscopy image of the miniscrew selected in this study. a) Part of the mini-screw inserted in the alveolar bone; b) part of the mini-screw exposed to the mouth corrosive environment

Fig. 2. Distribution of % DNA in tail found in buccal mucosa cells of all patients analyzed.

Fig. 3. DNA damage in oral mucosa cells of the control group, orthodontic group, orthodontic-miniscrew group, and positive control group (smokers). The levels of DNA strand-breaks are expressed as % DNA in tail. All values are expressed as mean ± s.d. *** significantly different from control group ($p < 0.001$). ### positive control group in comparison to orthodontic group ($p < 0.001$). ‡‡‡ positive control group in comparison to orthodontic-miniscrew group ($p < 0.001$).

Fig. 4. DNA damage in oral mucosa cells and their relation with the sex of subjects. The levels of DNA strand-breaks are expressed as % DNA in tail. All values are expressed as mean ± s.d. The significance levels observed are *** $p<0.001$ and *$p<0.05$ in comparison to control group values.

Fig. 5. Typical buccal cell comet images from control group (a), orthodontic group (b), orthodontic-miniscrew group (c) and positive control group (d) following lysis at pH 10, pre-treatment with proteinase K and electrophoresis in 1mM EDTA and 0.3 M NaOH for 20 min at 25 V.

CAPÍTULO 6 / CHAPTER 6

Ana Martín-Cameán, Angeles Jos, Pilar Mellado-García, Alejandro Iglesias-Linares, Enrique Solano, Ana M. Cameán

IN VITRO AND IN VIVO EVIDENCE OF THE CYTOTOXIC AND GENOTOXIC EFFECTS OF METAL IONS RELEASED BY ORTHODONTIC APPLIANCES: A REVIEW

Environmental Toxicology and Pharmacology (en revisión) **2015**

Elsevier Editorial System(tm) for Environmental Toxicology and Pharmacology
Manuscript Draft

Manuscript Number:

Title: In vitro and in vivo evidence of the cytotoxic and genotoxic effects of metal ions released by orthodontic appliances: A review

Article Type: Review Article

Keywords: Orthodontic appliances, metal release, cytotoxicity, genotoxicity

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Abstract: Intraoral fixed orthodontic appliances are frequently used in the clinical practice of dentistry. They are made from alloys containing different metals at various percentages. The use of these appliances leads to the long-term exposure of patients to these materials, and the potential toxic effects of this exposure raises concerns about patient safety. Thus, the biocompatibility (corrosion behaviour and toxicity) of these materials has to be evaluated prior to clinical use. In the present report, the most recent studies in the scientific literature examining metal ion release from orthodontic appliances and the toxic effects of these ions have been reviewed with a special focus on cytotoxicity and genotoxicity. Previous studies suggest that a case-by-case safety evaluation is required to take into account the increasing variability of materials, their composition and the manufacturing processes. Moreover, in vivo toxicity studies are still scarce. Further investigations could be performed to elucidate the toxic mechanisms involved in the observed effects with a special emphasis on oxidative damage. Additionally, in vitro and in vivo monitoring studies are needed to establish cause-effect relationships between metal ion release and biomarkers of cytotoxicity and genotoxicity.

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***Cover Letter**

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Dear Editor,

I would be very grateful if you consider the article entitled:

***"In vitro* and *in vivo* evidence of the cytotoxic and genotoxic effects of metal ions released by orthodontic appliances: A review"**

for its publication in the journal "Environmental Toxicology and Pharmacology**".**

The authors of the article were: Ana Martín-Cameán[1*], Ángeles Jos[2], Pilar Mellado-García[1] , Alejandro Iglesias-Linares[3], Enrique Solano[1], Ana M. Cameán[2].

The corresponding author's is: Ana Martin-Cameán

In the present report, the most recent studies in the scientific literature examining metal ion release from orthodontic appliances and the toxic effects of these ions have been reviewed with a special focus on cytotoxicity and genotoxicity. Previous studies suggest that a case-by-case safety evaluation is required to take into account the increasing variability of materials, their composition and the manufacturing processes. Moreover, *in vivo* toxicity studies are still scarce. Further investigations could be performed to elucidate the toxic mechanisms involved in the observed effects with a special emphasis on oxidative damage. Additionally, *in vitro* and *in vivo* monitoring studies are needed to establish cause-effect relationships between metal ion release and biomarkers of cytotoxicity and genotoxicity.

Looking forward to hearing from you,

Ana Martín-Cameán

In vitro and *in vivo* evidence of the cytotoxic and genotoxic effects of metal ions released by orthodontic appliances: A review

Concise title: Cytotoxicity and Genotoxicity of orthodontic appliances: A review

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Abstract:

Intraoral fixed orthodontic appliances are frequently used in the clinical practice of dentistry. They are made from alloys containing different metals at various percentages. The use of these appliances leads to the long-term exposure of patients to these materials, and the potential toxic effects of this exposure raises concerns about patient safety. Thus, the biocompatibility (corrosion behaviour and toxicity) of these materials has to be evaluated prior to clinical use. In the present report, the most recent studies in the scientific literature examining metal ion release from orthodontic appliances and the toxic effects of these ions have been reviewed with a special focus on cytotoxicity and genotoxicity. Previous studies suggest that a case-by-case safety evaluation is required to take into account the increasing variability of materials, their composition and the manufacturing processes. Moreover, *in vivo* toxicity studies are still scarce. Further investigations could be performed to elucidate the toxic mechanisms involved in the observed effects with a special emphasis on oxidative damage. Additionally, *in vitro* and *in vivo* monitoring studies are needed to establish cause-effect relationships between metal ion release and biomarkers of cytotoxicity and genotoxicity.

Keywords: Orthodontic appliances, metal release, cytotoxicity, genotoxicity

1. Introduction

Metal ion release during orthodontic treatment has become an important issue in the assessment of the biosafety of orthodontic treatments (Chojnacka and Mikulewicz, 2014). Most appliances routinely applied during orthodontic treatment are made of alloys that contain cobalt (Co), chromium (Cr), iron (Fe), nickel (Ni), and titanium (Ti). Among them, Ni and Cr have generated great concern (Mikulewicz and Chojnacka, 2010) because, in addition to their intrinsic toxicity, metal-based orthodontic appliances contain 8-50% Ni and 17-22% Cr on average. Well-conducted systematic reviews of the release of metal ions from orthodontic appliances through *in vitro* and *in vivo* studies have been previously carried out by Mikulewicz and Chojnacka (2010, 2011a).

Many of these metallic ions are essential trace elements and are absorbed with food. Nevertheless, a localized increase or a systemic distribution with increased deposits of these metals in another region may produce a toxic reaction (Rose et al., 1998). Ni and Cr, in particular, have been associated with hypersensitivity, cytotoxic and genotoxic effects (Amini et al., 2013). The extent to which a metallic substance is tolerated biologically depends on the quantity of liberated ions (i.e., the corrosion rate), the pathogenicity of the metallic ions released and the reaction products of the ions (Rose et al., 1998). Certain elements have an inherently higher tendency to be released from dental alloys based on to the lability of each metal. Copper (Cu) and Ni are labile elements (Wataha, 2000). The extent to which the released corrosion products can produce clinically relevant effects, both local and systemic, on the health of the patients, is not fully known (Grimsdottir and Hensten-Pettersen, 1993; Rose et al., 1998; Wataha, 2000; Geurtsen, 2002; WesTphalen et al., 2008). Moreover, routine clinical use and manipulation of these orthodontic appliances may interfere with the properties of these materials and their biocompatibility. Due to potential toxic effects, the biocompatibility of orthodontic materials has to be evaluated before their clinical use. There are different reports available in the scientific literature on this topic, including a systematic review on the cytocompatibility of medical biomaterials (Mikulewicz and Chojnacka, 2011b). Moreover, the American Society for Testing and Materials (ASTM) and the International Organization for

Standardization (ISO) have standards that cover specifically dental materials (ISO 7405) and medical devices (ISO 10993), which also include dental materials.

In the present report, three main aspects regarding the biocompatibility of orthodontic materials have been reviewed: 1) current knowledge on the data of metal ion release from orthodontic appliances, 2) the main toxic effects associated with ion metals frequently present in orthodontic materials and 3) cytotoxicity and genotoxicity studies performed on buccal mucosa cells exposed to metal materials. The main objectives were to identify state-of-the-art information about these points and to identify possible data gaps and research needs in this field.

2. *In vitro* and *in vivo* studies on the release of metal ions from orthodontic appliances and their toxicological implications

2.1. *In vitro* studies

In the systematic literature review of the release of metal ions under *in vitro* conditions published by Mikulewicz and Chojnacka (2011a), the authors concluded that the most popular assays are batch tests in which the materials are submersed in an environment of artificial saliva, 0.9% NaCl, or organic acids (such as lactic acid) and the released metal ions are quantified by different techniques, such as atomic absorption spectroscopy (AAS), inductively coupled plasma optical emission spectrometry (ICP-OES), or inductively coupled plasma mass spectrometry (ICP-MS), throughout the experiment and/or at the end of the experiment. In general, the advantages of these *in vitro* tests are simplicity, elimination of other factors that are not related to the orthodontic treatment, reproducibility, and lack of ethical problems (Chojnacka and Mikulewicz, 2014). The main disadvantage of *in vitro* tests is that they do not reflect the real conditions of the oral cavity (e.g., the presence of biofilms, which grow on the surface of the materials) and that the materials may respond differently under laboratory conditions versus clinical conditions (Mikulewicz and Chojnacka 2011a). Many of these *in vitro* studies use different methodological/material approaches, including various

types of immersion liquids, different analytical methods, and materials obtained from several manufacturers. All of these factors make comparing studies more difficult, or even impossible (Mikulewicz et al., 2014a). Therefore, Mikulewicz and Chojnacka (2011a) recommended that procedures be standardized (including the type and volume of immersion media, incubation conditions, static/dynamic conditions, and the duration of the experiment) to obtain results that are potentially comparable.

In the present work, Table 1 summarizes more recent *in vitro* studies on released metals from fixed orthodontic appliances, including information on the orthodontic material employed, the solutions used, the duration of treatment, the method of sample collection, the instrumental technique chosen for measuring metal ions, the median or mean concentrations of trace metals investigated and the main findings. These new *in vitro* studies were performed with different goals: 1) to compare the biocompatibility of different orthodontic materials as a function of released metallic ions (Suarez et al., 2010; Ortiz et al., 2011; Mikulewicz et al., 2012); 2) to investigate the potential influence of several typical treatments for the patient (i.e., use of mouthwashes) on metal release (Danaei et al., 2011); and 3) to monitor changes in metal release after the application of new treatments or laboratory methods that could improve the biocompatibility of orthodontic appliances, such as oxidation treatments (Espinar et al., 2011), different recycling techniques (Reimann et al., 2012), the effects of thermocycling and pH of orthodontic archwires (Sheibaninia, 2014), or differences between orthodontic bands with or without silver-soldered joints (Gonçalves et al., 2014). All these experiments aimed to understand the dominant factors that could affect corrosion resistance or adversely affect the release of elements. However, as far as we know, no standardization of these studies has been carried out. Consequently, the problem of comparing the data between studies has yet to be solved.

A brief description with the most important findings of these studies is shown below.

Suárez et al. (2010) investigated Ni released in 3 types of lingual orthodontic archwires (stainless steel (SS), NiTi and CuNiTi) after 7, 14 and 30 days of immersion in saline solution. They

demonstrated that SS archwires (8% Ni) released the highest amount of Ni compared to NiTi and NiTiCu archwires (which both have 50% Ni content). This result was also reported previously by Hwang et al. (2001). In all conditions, the Ni amounts released were lower than 1/10 of the Ni required to induce cell damage. Similarly, Ortiz el al., (2011) compared the amount of metallic ions released by 3 alloys (stainless steel, nickel-free, and titanium) in a culture medium over 30 days, and their results reinforce the idea that materials with higher Ni content are more susceptible to corrosion because materials with high Ni content release greater quantities of Ni, Fe, Cr, and Mn into the culture media during the first week of immersion. The titanium brackets and tubes were the most biocompatible of the 3 alloys that were investigated. Sfondrini et al. (2010), whose results agreed with the findings of previous studies (Huang et al., 2003; Huang et al., 2004), showed that the release of Ni from three types of brackets (new conventional SS, recycled SS, and Ni-free brackets) was higher for recycled brackets, whereas the lowest release of Ni was from Ni-free brackets. This result could be explained by the high temperatures used to heat steel during recycling. A chromium carbide precipitate is formed under high temperatures, and that precipitate is susceptible to intragranular corrosion, which likely leads to general weakening of the structure. Moreover, for all types of brackets, acidic conditions (pH 4.2) produced the highest Ni release, an outcome similar to results obtained by Milheiro et al. (2012), and the high release may have occurred because acidic conditions provide a reduced environment in which the stainless steel oxide film required for corrosion resistance is less stable (Huang et al., 2003). A significant increase in Ni release was also demonstrated as a function of the immersion period for all of the brackets, except for the time interval between 24-48 hours. In contrast, Gil et al. (2012) reported a significant reduction in Ni ion release from recycled NiTi archwires in the saliva compared to original archwires. These authors found a reduction in Ni content in the matrix of these archwires after thermal treatment due to the formation of very stable Ti_3Ni_4 precipitates. For both types of archwires, the concentration of ions released in the medium initially increased very fast, but later in the experiment, the concentration was saturated. This saturation has also been reported by the same authors, who demonstrated a

reduction in Ni release from NiTi archwires treated with an oxidation treatment designed to obtain Ni-free surfaces. The treatment resulted in the formation of a titanium oxide protective layer (Espinar et al., 2011).

Because NiTi wires are subjected to mechanical stresses and deformation from tooth movement, the applied forces might induce damage of the oxide film on the surface of the wires and subsequent loss of protection. Therefore, Liu et al. (2011) measured Ni release under a continuous bending stress to simulate the intraoral environment, and they demonstrated that bending stress induced greater Ni ion release from NiTi wires compared to unstressed archwires. They suggested that bending stress induced damage of the passivated oxide film on NiTi wires, and the release of ions could be explained by the exposed active metal surface rather than metal-ion transport through the oxide film and hydrolysis of the oxides. Consequently, stress is an important factor to be considered on the corrosion behaviour in the design and clinical use of these NiTi wires.

Orthodontic space maintainers were studied by Bhaskar and Subba Reddy (2010), who showed in their study that the release of Cr and Ni ions from bands reached maximum quantities at the end of 7 days. Nevertheless, these authors did not find significant differences in ion release when different band materials were used in the bands of these space maintainers.

Danaei et al. (2011) studied the release of Cr, Cu, Fe, Mn and Ni from SS orthodontic brackets after treatment with 3 different mouthwashes was found to reduce the risk of white-spot lesions around the brackets. After 45 days of immersion, they concluded that the highest ion release occurred in the presence of chlorhexidine mouthwash, and they recommended avoiding prolonged application of chlorhexidine mouthwash in patients who have allergies.

The reconditioning of orthodontic brackets by different techniques, including direct flaming, acid bath recycling, or commercial recycling, can degrade the most essential properties of these materials, even though significant differences were not observed in nickel-ion release after static immersion of reconditioned brackets in Fusayama's artificial saliva (Reimann et al., 2012). Recently, Sheibaninia (2014) evaluated the effect of pH changes and thermocycling, two main factors that

change many times during the day after each meal and drink, on the amount of Ni released from orthodontic appliances. According to the results, thermocycling adversely affected the release of Ni from NiTi wires, and an acidic environment accelerated the rate of Ni ion release. The results showed that pH had some influence on Ni release, but thermocycling was clearly the dominant factor.

The release of Cu ions has been investigated very little in comparison to Ni. Recently, Zhang et al. (2014) reported that composite archwires (CoAW), formed by solder connection on a NiTi shape memory alloy and stainless steel wire, were resistant to corrosion in simple artificial saliva. The CoAW showed the greatest weight loss and Cu release in chloric solution. The corrosion resistance of the Cu interlayer depends primarily on the oxidation product formed on the interlayer surface after the initial corrosion. According to these authors, damage to the oxide film would allow active metal to react with the surrounding environment and cause further ion release. However, the greatest level of Cu release detected was lower than the Provisional Maximum Tolerable daily Intake (PMTDI) limit established for this metal.

A complete *in vitro* study on the release of 17 elements from stainless steel (SS) orthodontic appliances has been carried out by Mikuliewicz et al. (2012). Their study reinforced the idea that Ni and Cr are metals that require more attention. Moreover, in this study, three ion release coefficients were defined: α (dimensionless), which give the degree of release of metal ions; β, which is the difference between the concentrations (μg/L) of ions in artificial saliva relative to the control solution; and γ, which is the ion release coefficient (%). These coefficients were used to show which elements were dissolved to the highest extent and to demonstrate that the release of these metals was proportional to their content in the alloy. The concentrations of Ni and Cr were also correlated, which suggests that these ions were released together as a result of corrosion. When these concentrations were compared to the maximum admissible concentrations or recommended levels, the researchers concluded that the only possible risk for orthodontic patients would be exposure to nickel.

In a later study, the same authors designed a new continuous-flow system for the *in vitro* testing of fixed orthodontic appliances (thermostatic glass reactor) to evaluate the release of metal ions (Mikulewicz et al., 2014a). Compared to previous investigations, this innovative study incorporated an approach that is a greater approximation of *in vivo* assays due to continuous monitoring of ion release over a prolonged period of time. The experimental conditions reflected the human oral cavity (37ºC), and the saliva flow rate was 0.5 mL/min. Sampling was performed at several time points during the 28-day study, and the metal ion concentrations of eight elements were determined by ICP-OES. The ions were released in the following order: Si>Cu>Ni>Cr>Mo>Mn>Cd. Fe ions were not released from the appliance, and there was no correlation between the content of a given metal in an alloy and the released quantity. Statistically significant differences were found for Ni, Cr, and Cu between the experimental and control groups. The highest concentration of metals was released at the beginning of the experiment. After this, point passivation probably occurred, which hindered further release. The total mass of released metal ions over 4 weeks was as follows: Ni (18.7 μg), Cr (5.47 μg), and Cu (31.3 μg). All the values were far below the recommended daily doses, so the treatment might not be a significant source of exposure to these metal ions.

2.2. *In vivo* studies

Regarding *in vivo* studies, previous investigations have demonstrated the corrosion and release of metal ions from orthodontic appliances through emission of electro-galvanic currents with saliva acting as the medium for continuous erosion over time (Kerosuo et al., 1997; Kocaderelli et al., 2000; Agaoglu et al., 2001; Eliades et al., 2003; Amini et al., 2008; Matos de Souza et al., 2008; Petoumeno et al., 2008). These results were systematically reviewed by Mikuliewicz and Chojnacka (2010). Most of these experiments measured ion release (especially Ni, Cr, Co, Fe, Ti, Mo) during exposure to a biological medium, such as blood, serum, urine, or saliva, for periods ranging from 1 day to 1-2 months. In general, an increase in the salivary concentration of Ni and Cr followed the

insertion of fixed orthodontic appliances (Kocadereli et al., 2000; Eliades et al., 2003; Fors and Pearson, 2006). The general conclusions of these studies (as of 2010) were that metal ions are released only in the initial stage of the orthodontic treatment. Moreover, there is a lack of *in vivo* long-term studies that monitor chronic exposure over several years of therapy (each orthodontic treatment lasts 24-30 months). Additionally, Mikuliewicz and Chojnacka (2010) indicated that no research using hair or nail analysis had been reported, although these techniques can be useful for biomonitoring of exposure from dental appliances. Moreover, different authors agree that *in vivo* investigations are urgently needed to study the behaviour and biocompatibility of different commercially available dental alloys under real-life conditions (Matusiewicz et al., 2014).

With respect to the new data reported by the *in vivo* studies reviewed in this work (Table 2), some research has focused on the recommendations to investigate new matrices and long-term exposure. Several studies on saliva confirmed once more that Cr and Ni levels found in this matrix should be monitored because of their toxicity. Amini et al. (2012) found a significant increase in Ni levels in the saliva of patients treated with stainless steel (SS) arches, but they did not find an increase in Cr levels. Later, the same authors (Amini et al., 2013) were the first researchers to evaluate the effect of stress on salivary Cr and Ni contents in orthodontic patients at different times. They concluded that induction of stress led to a significant increase in Ni release into saliva, whereas Cr content was not significantly altered. Freitas et al. (2011) evaluated the toxicity of silver solder in orthodontics by measuring the release of Ag, Cd, Cu, and Zn in the saliva of control and experimental groups at 6 time points: before placement of the appliance and 10 min, 24 h, 7 days, 30 days and 60 days after placement. In the experimental group, significant concentrations of all elements were detected 10 min after placement. The highest mean concentration was found for Cu, and the lowest mean concentration was found for Zn. Comparison between the groups revealed significant differences for Cu (all periods), Zn (10 min, 24 h, 7 days and 30 days), and Cd (10 min). The researchers concluded that high concentrations of ions were released after placement of the

appliances and the metals with risk for potential toxicity, in decreasing order, were Cu, Ag, Cd and Zn. In this first *in vivo* study, which lasted for 60 days, reductions in metal concentrations were observed after 30 and 60 days. The tendency of the concentrations to stabilize and return to the values measured before placement of the appliances was associated with the chemical passivity reached by the metals in the mouth from biofilm formation that alters the surface characteristics of the alloy.

Despite of the advantages of saliva over blood collection (non-invasive, lower cost, no risk of infection, no special handling or preservation conditions required), the matrix used in this *in vivo* study has some disadvantages: its flow is influenced by many factors, and it only provides information about the moments in which the samples were collected (Hafez et al., 2011). Oral mucosa cells, with prolonged contact with fixed appliances, could function as an alternative matrix, but this approach has not been investigated fully (Amini et al., 2008; Faccioni et al., 2003; Hafez et al., 2011; Natarajan et al., 2011; Fernandez-Miñano et al., 2011). Recently, two methods were developed and validated in our laboratory, which included robustness studies, for the simultaneous determination of vanadium (V), titanium (Ti) and zirconium (Zr) release (Martin-Cameán et al., 2014a), or Co, Cr, Cu and Ni release (Martin-Cameán et al., 2014b) in buccal mucosa cells. No significant differences in Ti concentrations were detected in orthodontic patients compared to control subjects. Only traces of Zr were detected and vanadium was not detected in either the orthodontic group or the control group. The mean values of Co, Cr, Cu and Ni significantly increased in the orthodontic group. More studies using this matrix are necessary to confirm these data and to acquire additional information about the *in vivo* corrosive potential of intraoral appliances, which should include studies that monitor appliances throughout the orthodontic treatment.

In addition to using conventional biological samples to evaluate metal release from orthodontic appliances, some recent studies have demonstrated that human hair is an adequate non-invasive matrix for monitoring different ion metals (Mikulewicz et al., 2011c; Martin-Cameán et al., 2014c; Mikulewicz et al., 2015). Human hair is a stable matrix that presents numerous advantages

for human biomonitoring, including easy collection, low cost, easy transport and storage. Hair samples also do not show storage changes from the period between sampling and analysis, and hair samples provide information about short- and long-term exposure (Gil et al., 2011), including the temporal exposure pattern calculated through segmental analysis (Esteban and Castaño, 2009). The first attempt to use human hair to investigate metal release from fixed orthodontic appliances was carried out by Mikulewicz et al. (2011c). This preliminary study (28 patients/18 controls) demonstrated that stainless steel appliances were the source of significant exposure to Ni and noted that 22% of patients undergoing orthodontic treatment had increased Ni levels in their hair. The researchers applied the coefficients α_{hair} and β_{hair} in their assessment of the bioavailability of released metal ions in patients. The highest difference between the groups was found for Ni (39%), Mn (18%), Fe (4.1%) and Cr (2.5%). Furthermore, statistically significant correlations were found between Cr and Fe, which showed that these metals had similar sources of exposure, and multiple regression analysis determined the dependence of Ni content on the level of Co and Mg (synergism) and V (antagonism).

Later, a study from our laboratory in human scalp hair from a broad population group with orthodontic appliances (n=70) confirmed that hair mineral analysis is a good method for investigating long-term exposure to different elements (Cr, Cu, Fe, Mn and Ni) (Martin-Camean et al., 2014c). In orthodontic patients, differences in the content of metals in hair were only significantly increased for Mn compared to the control group, but their levels were of the same magnitude to other control populations, and no risks linked to the treatment were found. The accumulation of metals in hair was higher in females, and only Mn levels decreased with age in the orthodontic group. Moreover, correlations found between Cu/Mn, Cu/Ni and Fe/Ni showed a mutual dependence of these elements in hair (similar chemical structure). Mikuliewicz et al. (2015) evaluated metal ion accumulation in the hair of patients with fixed appliances at different time points throughout their treatment (the beginning and in the 4^{th}, 8^{th}, and 12^{th} months of the treatment). These authors reported a peak release of Cr and Fe after 4 months, and the Ni peak gradually increased throughout the year. This study revealed that the Cr content was significantly higher during the treatment,

although the doses of the released metal ions did not pose toxicological risks, and the researchers demonstrated that hair mineral analysis permits the study of the kinetics of metal ions and ion transfer to hair tissue.

Other innovative work has focused on the utility of invasive matrices in *in vivo* experimental models, such as pigs, to study the accumulation of metal ions released from orthodontic appliances after long-term exposure (Mikulewicz et al., 2014b). Hair (non-invasive) and kidney, liver, lung, aorta, and oral mucosa cells were collected from experimental pigs (with plates) and control pigs for multi-elemental analysis. The greatest differences were found in the aorta (Ni levels were 4.8 times higher in the experimental group vs. the control group), in the cheek (Ni levels were 3.5 times higher) and in the hair sampled after 3 months. Metal ions were released from the appliances in low doses, especially at the beginning of the experiment, and the doses did not reach toxic levels.

The kinetics of metal ions released under *in vitro* (artificial saliva) and *in vivo* conditions (pig, human hair) have been recently discussed using a mathematical function (Chojnacka and Mikulewicz, 2014). The authors defined the ω coefficient to combine the *in vitro* and the *in vivo* data on animals on one hand with the in *vitro* and the *in vivo* data from human beings. The model was positively verified in pigs, and it can be extrapolated for use on patients to determine the dose of metal ions that patients are exposed to during orthodontic treatment. This matter is of great interest, and further studies are needed to develop tools that allow researchers to extrapolate the data obtained from *in vitro* and *in vivo* procedures to clinical practice.

2.3. General toxic effects of metal ions released from orthodontic appliances

The potential implications for human health derived from the release of ions from metallic orthodontic appliances are still a matter of substantial concern. In general, most of the *in vivo* studies that have evaluated the discharge of metal ions from orthodontic appliances in biological fluids (section 2.2) have concluded that the levels of metal ions do not reach the normal daily dietary

intake of some elements, such as the Tolerable Daily Intake (TDI) of 2.8 µg/kg body weight/day for Ni (EFSA, 2015; http://www.efsa.europa.eu/en/search/doc/4002.pdf), the estimated maximum intake for Co (39 µg/d) (EGVM, 2003; http://cot.food.gov.uk/sites/default/files/cot/vitmin2003.pdf)), the Tolerable Upper Intake Level (UL) established for Cu (5 mg/day), or Cr trivalent (1 mg/day) (SCF, 2003a,b). However, the possibility remains that even nontoxic concentrations of cations released from dental alloys might be sufficient to produce biological alterations (e.g., in DNA synthesis or alkaline phosphatase activity) (Geurtsen, 2002). Occasionally, the host response to the elemental release differs according to the nature of and amount of the released elements. Classical allergic responses are characterized by dose-independence, i.e., low doses would not cause inflammation through toxicity but would activate immune cells (Schmalz et al., 2000). Additionally, mutagenicity and carcinogenic effects are not related to the dose of the toxicant. However, to understand dose-dependent toxic effects, it is necessary to know the bioavailability of the metallic ions of interest. In this regard, the scientific literature suggests that the oral bioavailability is low and ranges between 0.4-2.5% for Cr or between 30-40% for Cu (SCF, 2003). The extent of gastrointestinal absorption of Co depends upon the dose (EGVM, 2003). Moreover, it is also important to know if the released elements could generate localized toxicity adjacent to the restoration (Wataha, 2000).

The main elements involved in corrosion products are Ni, Cr, and Fe from stainless steel and Ti and Ni from NiTi alloys. Ni and Cr ions are of particular interest because of their allergenic properties, especially Ni, and these ions have mutagenic, cytotoxic and carcinogenic effects, which are briefly summarized below.

Regarding Ni, the percentage of Ni in metallic alloys used in orthodontic treatments ranges from 8% (stainless steel) to more than 50% (NiTi alloys) (Railly and Price, 2003). Diverse cases of intra- or extraoral allergic reactions after exposure to Ni-containing orthodontic appliances have been reported over the last 30 years (Volkman et al., 2007; Noble et al., 2008). Ni is a strong immunologic sensitizer that causes allergic contact dermatitis, which is a Type IV delayed

hypersensitivity immune response occurring at least 24 hours after exposure. Tissue reactions to Ni may consist of intraoral diffuse red zones, hyperplasia, change in colour, bleeding, blisters and ulcerations extending to the perioral area, and eczematic as well as urticarial reactions of the face or more distant skin areas that are sometimes severe (Pazzini et al., 2009; Kolokitha and Chatzistavrou, 2009; Menezes and Quintao, 2010). Whereas oral exposure to Ni is not known to lead to sensitization, oral absorption of Ni can elicit eczematous flare-up reactions in the skin in Ni-sensitized individuals. Concentrations as low as 8 and 12 μg Ni/kg body weight have provoked such reactions (EFSA, 2006; 2015). However, the literature investigating the relationship between Ni-allergy and orthodontic appliances is controversial, with prevalence of the allergy ranging between 17.2% (Pazzini et al., 2009) to 0.03% (Volkman et al. 2007). In general, although adverse reactions in patients with Ni-allergy have been observed, they are uncommon, and risks of sensitization have been considered insignificant (Menezes et al., 2004; Setcos et al., 2006; Kolokitha et al., 2008). Recent clinical evidence suggested that treatment with Ni-containing metallic orthodontic appliances before sensitization to the metal (e.g., ear piercing) may reduce the frequency of Ni hypersensitivity from full fixed appliances and the length of treatment by a factor of 1.5-2. These effects support the concept of inducing immunological tolerance via oral administration of Ni (Fors et al., 2012).

Moreover, Ni is known to have cytotoxic effects, probably due to increased production of intracellular lactate dehydrogenase, which affects the redox equilibrium and stimulates apoptosis in *in vitro* oral human epithelium cells (Trombetta et al., 2005). Regarding the molecular mechanisms of toxicity, oxidative stress and inhibition of Bcl-2 expression are involved in Ni-induced apoptosis (Rana, 2008).

Some Ni-compounds have been considered as carcinogens, and the International Agency for Research of Cancer (IARC) evaluated and classified Ni compounds as group 1 carcinogens for humans (http://monographs.iarc.fr/ENG/Monographs/vol100C/mono100C-10.pdf). The exact mechanisms of Ni-induced carcinogenesis are not known because Ni compounds are weakly mutagenic (in comparison to other metals) in most assays, and Ni compounds are likely involved in genetic and

epigenetic routes. In addition to chromosomal damage, DNA-protein cross-links and oxidative DNA base damage are observed in Ni(II)-exposed cells, and Ni also inhibits DNA repair. Nickel induces oxidative stress that depletes glutathione and activates AP1, NF-*k*B and other transcription factors sensitive to oxidation (Denkhaus and Salnikow, 2002). Moreover, the epigenetic effects of Ni include alterations in gene expression, which are induced by DNA hypermethylation and suppression of histone acetylation, as well as activation or silencing of certain genes and transcription factors (Denkhaus and Salnikow, 2002; Kasprzak et al., 2003). Because the Ni-containing alloys in orthodontic appliances are reported to be not carcinogenic, the risks of genetic damage are considered to be minimal, and no reports of carcinogenicity associated with the intraoral use of these dental alloys have been found (Wataha, 2000; Setcos et al., 2006). Other studies have suggested that chronic exposure to Ni-containing dental materials may adversely affect human monocytes and may affect cytokine secretion indirectly by modulation of the Nrf2 antioxidant pathway (Lewis et al., 2006).

The oxidation state of chromium (Cr) has been linked to genotoxicity both *in vivo* and *in vitro* (Beyersmann and Hartwig, 2008). The state of Cr is critical to its mutagenic activity because only Cr (IV) is a mutagen (Wataha, 2000). Structural genetic lesions produced by the intracellular reduction of Cr(VI) include DNA adducts, DNA stand breaks, DNA-protein crosslinks, oxidized bases, abasic sites, and DNA inter- and intrastrand crosslinks. The damage can lead to dysfunctional DNA replication and transcription, aberrant cell cycle checkpoints, deregulated DNA repair mechanisms, microsatellite instability, inflammatory responses, and the disruption of key regulatory gene networks responsible for the balance of cell survival and cell death. All these negative effects may play an important role in Cr (VI) carcinogenesis (Nickens et al., 2010; Gonçalves et al., 2014). Moreover, reactive oxygen species (ROS) and p53 contribute to apoptosis induced by Cr (VI) (Rana et al., 2008).

Copper ions can also be released in high amounts from some orthodontic appliances (bands), and an excess of Cu is involved in the formation of OH^- radicals generated from H_2O_2 via the Haber-Weiss and Fenton reactions. These radicals can initiate nonspecific lipid peroxidation (Gonçalves et al., 2014). Cu is also cytotoxic and induces apoptosis through p53-dependent and p53-independent pathways (Geurtsen, 2002; Rana, 2008).

Cobalt ions and Co metal are cytotoxic and induce apoptosis and, at higher concentrations, necrosis with an inflammatory response (Simonsen et al., 2012). The mechanisms of Co toxicity are not clear, but some effects could be related to the high affinity of Co for sulphydryl groups, which may cause inhibition of crucial enzymes. Co toxicity could also arise from the displacement of divalent cations in the ion centre of metal-activated enzymes, the effects of Co as a Ca^{2+} channel antagonist, and the generation of ROS in cells through Fenton-like reactions, which lead to oxidative stress and oxidative damage in proteins, lipids and DNA (Simonsen et al., 2012). This oxidative DNA damage together with alterations in DNA repair processes explains the genotoxic effects of Co metal and salts that have been reported in mammalian *in vitro* test systems. Carcinogenic effects have also been observed with Co (II), which increases the risk of cancer in humans, but no carcinogenicity data are available following exposure through an oral route (EGVM, 2003). As we have stated before, no studies have been published supporting the hypothesis that treatment with orthodontic appliances might be a human carcinogenic hazard (Geurtsen, 2002).

3. Cytotoxic and genotoxic effects of orthodontic appliances

New dental materials for clinical use (in orthodontics, endodontics, prosthetics and implantology) are considered medical devices, and their approval in the biomedical field depends on the biocompatibility of the materials. For that reason, investigations into the cytotoxicity and mutagenic/genotoxic potential of these materials by using a battery of tests from different models are of great interest (Velasco- Ortega et al., 2010).

3.1. Cytotoxicity studies

Cytotoxicity tests constitute an efficient first step in a biocompatibility study and reduce animal use in the laboratory (Assad et al., 1994). The term "cytotoxicity" is used to describe the cascade of molecular events that interfere with macromolecular synthesis and lead to unequivocal cellular, functional and structural damage (Aldridge, 1993; Murray et al., 2007). Regarding dental treatments, it is advantageous to maintain maximal tissue vitality and cytotoxic reactions must be prevented, which is why all dental compounds are required to be screened before they are used clinically (Murray et al., 2007). Table 3 shows the reports available in the scientific literature concerning the cytotoxicity of orthodontic materials from the last ten years. In the present review, only reports focusing on orthodontic materials have been considered. There is another excellent systematic review (Mikulewicz and Chojnacka 2011b) in which the cytocompatibility of medical biomaterials containing Ni with osteoblasts was evaluated. The present review did not apply limitations on the experimental model used, which allowed for a broad spectrum of studies in the field to be analysed.

The recommended testing methods (ISO 10993; ISO 7405) use cell counting, dye-binding, metabolic impairment or membrane integrity as end-points for the cytotoxicity test or assay (Murray et al., 2007). Among them, the MTT test is the most popular (Rose et al., 1998; Es-Souni et al., 2001; 2002; Mockers et al., 2002; Eliades et al., 2004; Nocca et al., 2006; Kao et al., 2007; Malkoc et al., 2010; Ortiz et al., 2011; Zhang et al., 2014; Gonçalves et al., 2014). This assay is based on the capacity of the cells to reduce the tetrazolium dye MTT 3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble formazan through the activity of the mitochondrial succinate dehydrogenase in living cells. Assays based on cell staining (e.g., with neutral red or trypan blue) are also frequently used (Tomakidi et al., 2000; Faccioni et al., 2003; Oh et al., 2005; Hafez et al., 2011; Bueno & Basting, 2014) as well as tests that evaluate morphological changes through observations under a microscope (Assad et al., 1994; Es-Souni et al., 2001; Assad et al., 2002; Bogdanski et al., 2002; Kobayashi et al.,

2007; Toy et al., 2014; Bueno & Basting, 2014). However, very few of the available reports in the scientific literature, which are shown in Table 3, refer to ISO/ASTM standards for performing the assays.

Several approaches have been used in addition to different methods to assay the cytotoxicity of orthodontic materials. Thus, some studies evaluated orthodontic materials in direct contact with cells, whereas other researchers exposed cells to eluates obtained after the orthodontic material was immersed in cell culture medium (or lactic acid, NaCl solution, etc.). The former studies, although they are closer to modelling real exposure because there is a direct contact between the cells and the material, only employ the short exposure times used in cytotoxicity tests (mainly 24-72 h). Therefore, the release of metals occurs in such a short time that these studies do not effectively model *in vivo* conditions. Using these methods, cytotoxicity cannot be directly correlated to either the types of ions or the concentrations of various ions released by the materials being tested (Tomakidi et al., 2000). However, the exposure of the cells to the highest levels of ions released by the test materials (using eluates obtained by immersing orthodontic materials in the proper medium for a certain amount of time) allows researchers to simulate the worst-case scenarios with cell cultures (Tomakidi et al., 2000).

The experimental models used to evaluate the cytotoxicity of orthodontic materials mainly include established cell lines of human and non-human origins. Regarding human cell lines, cell types found within the area of orthodontic appliance application (e.g., immortalized human gingival keratinocytes IHGK, fibroblast cells from explants of human gingival, and the human osteosarcoma cell line U2OS) and cells with no relation to orthodontic treatments (HepG2 from kidney, PK84 skin fibroblasts, etc.) are used as models. The non-human cell line that is frequently used is the L929 mouse fibroblast cell line (Assad et al., 1994; Rose et al., 1998; Mockers et al., 2002; Assad et al., 2002; Oh et al., 2005; Malkoc et al., 2010; Spalj et al., 2012; Zhang et al., 2014).

The results of these studies are quite variable. Some studies have reported cytotoxic effects of the tested materials (Assad et al., 1994; Es-Souni et al., 2001; etc.), whereas other investigations have demonstrated the biocompatibility of the tested materials (Wever et al., 1997; Ryhänen et al., 1997; Tomakidi et al., 2000; Assad et al., 2002; Zhang et al., 2014; etc.).

There is an additional way to assess the cytotoxicity of orthodontic materials. The approach consists of the extraction of epithelial buccal cells from orthodontic patients. In this approach, the viability of the cells is evaluated during or after orthodontic treatment (*in vivo* exposure). These studies are scarce, and the results are contradictory. Thus, Hafez et al. (2011) evaluated the cytotoxicity of buccal mucosa cells with the trypan exclusion test in 40 orthodontic patients and 20 control subjects before orthodontic treatment as well as 3 and 6 months after the appliance placement. They observed that cellular viability decreased, but this decrease was not evident at the 6-month time point, which possibly indicates that the cells were repaired or developed a tolerance for the appliance. Stainless steel brackets and archwires showed the least amount of biological damage, whereas Ti brackets and archwires produced the greatest cytotoxicity. Toy et al. (2004), however, found morphological evidence of cytotoxicity following 6 months of orthodontic treatment. Angelieri et al. (2011) observed that orthodontic therapy did not increase nuclear alterations after 6 months of treatment.

Cytotoxicity induced by orthodontic appliances is related to the metal ion release from corrosion processes. In this regard, approximately half of the studies in Table 3 also include analytical quantification of the metal ions found in orthodontic appliances, and there is a special focus on Ni content. The data on ion metal release from these materials have been reviewed in section 2.

All these studies suggest the importance of case-by-case safety evaluations of orthodontic appliances because the composition and the treatments performed on the materials have a role in the resulting cytotoxicity.

3.2. Genotoxicity studies

The genotoxic properties of metals from orthodontic appliances are an essential for determining the biological safety of these materials in patients (Montanaro et al., 2005). Genotoxicity tests can be defined as *in vitro* and *in vivo* approaches designed to detect compounds that induce genetic damage, including DNA lesions, gene mutation, chromosomal breakage, altered DNA repair capacity, and cellular transformation (Angelieri et al., 2011). Various orthodontic appliances (wires, brackets, extension screws) fabricated with different base metal alloys and cpTi were screened for genotoxic alterations, and these reports showed that various metals are genotoxic and/or carcinogenic. There is strong evidence that Ni, Co, Cr and Be increase the risk of cancer in humans (Geurtsen, 2002), although no studies have been published which support the hypothesis that dental casting alloys might be a carcinogenic hazard to man.

It is important to note that no single genotoxicity test is capable of detecting all relevant genotoxic agents. Therefore, in accordance with current regulatory requirements, medical devices are assessed for genotoxic potential with a battery of *in vitro* and *in vivo* genotoxicity assays. According to the International Standard ISO-10993, the following test battery is proposed to test genotoxicity: 1) a test for gene mutation in bacteria (Ames test, OECD 471, which is conducted with strains of *Salmonella typhimurium* and *Escherichia coli* designed to detect all possible single base pair changes as well as frameshift mutations); 2) an *in vitro* mammalian genotoxicity assay, such as one of the following recommended tests: a) the Mouse Lymphoma gene mutation assay (MLA) (OECD 476), which is preferred because it detects the broadest set of genotoxic mechanisms associated with carcinogenic activity, b) an *in vitro c*hromosomal aberration (CA) assay (OECD 473), or c) an *in vitro* micronucleus assay (MN) (OECD 487); and 3) an *in vivo* cytogenetics assay, such as one of the following recommended tests: a) a bone marrow micronucleus (MN) Assay (OECD 474), b) a bone marrow chromosomal aberration (CA) assay (OECD 475), or c) a peripheral blood MN assay.

Table 4 compiles the different *in vitro* and *in vivo* genotoxicity assays available in the scientific literature. The data from related research suggest that studies on the genotoxicity and DNA

damage resulting from orthodontic materials are rare. Regarding *in vitro* studies, some of the assays mentioned above have been utilized in research studies, such as the *Salmonella* reverse-mutation test (Wever et a., 1997; Montanaro et al., 2005), the chromosomal aberration (CA) test (Wever et al., 1997; Montanaro et al., 2005), and testing for sister chromatid exchanges (Montanaro et al., 2005), but the authors did not specify the use of OECD protocols. Moreover, the alkaline version of the single cell gel (comet) assay (Tomakidi et al., 2000; Angelieri et al., 2011), including the modified version using endonuclease III (Endo III) and formamidopyrimidine glycosylase (FPG) (Gonçalves et al., 2014), has also been used by researchers.

Below, detailed information on the results and conclusions stated in the genotoxic studies compiled in this review is provided.

In one of the pioneering *in vitro* studies, the researchers investigated the short-term biological safety of the NiTi alloy (Weber et al., 1997) used in wires for orthodontic tooth alignment as well as orthopaedic and cardiovascular applications. The previous biocompatibility results were conflicting. Good results were found using this material in two genotoxicity tests: the *Salmonella* reverse mutation test (with four *S. typhimurium* strains) in the presence and absence of metabolic activation, and the CA test in mammalian cells. However, the long-term biological safety and the biological response of corrosion products from the NiTi alloy need to be performed. Later, Angelieri et al. (2011b) evaluated the potential genotoxicity of corrosion eluates from four different commercial orthodontic brackets (after 1-70 days of submersion) with a comet assay in mammalian cells (CHO). They concluded that the brackets did not induce genetic damage. Tomakidi et al., (2000) investigated potential DNA damage from Ni-free wires, three types of brackets and a titanium expansion screw by using the alkaline comet assay on immortalized human gingival keratinocytes (IHGK). They observed no DNA strand breakage in cells (tail moment) in cultures exposed to the various eluates. The tests (cytotoxicity and comet assays) were carried out using monolayer cultures. Therefore, the results only demonstrate local effects. Consequently, further work on subtoxic effects

(such as inflammation, allergic response) is necessary to a better evaluate the biocompatibility of these metallic orthodontic materials.

The sensitivity and specificity of the comet assay can be improved by incubating lysed cells with lesion-specific enzymes, endonuclease III (Endo III) and formamidopyrimidine DNA glycosylase (FPG), which allows for the measurement of oxidised pyrimidines and oxidised purines, respectively (Collins, 2004). With these modifications, the comet assay has become a popular method for measuring various types of DNA damage, including oxidative damage cause by ROS (Collins, 2014; Llana-Ruiz-Cabello et al., 2014). In orthodontics, this enzyme-modified comet assay has been employed in research only once (Gonçalves et al., 2014) to assess the induction of oxidative DNA lesions from different stainless steel bands with silver soldered joints (SSB) or without silver soldered joints (NSB) or) in HepG2 (human hepatocellular carcinoma) and HOC (human oral keratinocyte) cell lines. Both bands released elevated levels of toxic metals, and the eluates from these bands increased the number of DNA breaks after both enzyme treatments, which suggest that these bands induced oxidative lesions in a similar manner. Because NSB eluates were less genotoxic than SSB eluates, the higher damage frequency observed in the SSB group without enzyme treatment could be related to a combination of oxidative DNA damage (which may be related to the interference of Cr and the other release metals, including Fe, Zn and Ni) and other direct effects of the metals detected in the culture medium. Moreover, in this study, the authors employed a human oral keratinocyte cell line for the first time to investigate the induction of genome instability using the cytokinesis-block micronucleus cytome (CBMN-cyt) assay in which cells are scored cytologically for their viability as well as their mitotic and genomic instability (Fenech, 2007). In this test, the SSB bands induced a significant increase in nucleoplasmic bridge formation, which is a sensitive biomarker that provides direct evidence of genome damage from mis-repaired DNA breaks or telomere to telomere end fusions related to metal ion release.

Taking into account the scarce number of studies published on orthodontic appliances, further studies with individual tests and a battery of the assays recommended by the International

standards are needed because these investigations will help us to evaluate and accurately assess the genotoxic potential of orthodontic applications. It is important to note that, to the best of our knowledge, the mouse lymphoma tk (MLA, OECD 476) assay has not been used yet.

As we have previously discussed, the *in vitro* data do not necessarily reflect *in vivo* clinical conditions because *in vitro* methods do not always simulate the conditions of an oral cavity with a multifactorial environment (Hafez et al., 2011). Current *in vivo* human studies are aimed at representing the real condition of the oral cavity by sampling buccal cells, which are directly exposed to the appliances (Faccioni et al., 2003; Westphalen et al., 2008). Thus, further biocompatibility data from *in vivo* human studies are needed to evaluate all the risks of orthodontic appliances (Angelieri et al., 2011).

Among *in vivo* tests, the comet assay (or alkaline single cell gel electrophoresis), which measures single- and double-strand breaks and alkali-labile sites in each individual cell, is considered to be a quick, simple, sensitive, reliable, and fairly inexpensive way of measuring DNA damage (Collins et al., 1997; Rojas et al., 2000). The comet assay is useful because it requires only a very small numbers of cells and it can be used to evaluate DNA damage in either proliferating or non-proliferating cells (Hartmann and Speit, 1994). The published protocols used in some reports of buccal cells, which are collected easily from the inside of the mouth, showed massive sustained damage and disintegration at the high pH used in the study. This protocol was improved by Szeto et al., (2005), who demonstrated that their buccal cell comet assay was a feasible and potentially useful alternative tool to the usual lymphocyte model employed in human biomonitoring and nutritional work to monitor the effects of lifestyle choices and chemical agents (Dusinska and Collins, 2008; Collins et al., 2014).

The comet assay and the Micronucleus (MN) assay (Westphalen et al., 2008; Natarajan et al., 2011; Öztürk et al., 2012; Heravi et al., 2013; Toy et al., 2014) are probably the most widely used

tests in *in vivo* studies (Table 4) that have been applied to orthodontic brackets and wires (Faccioni et al., 2003; Westphalen et al., 2008; Fernández-Miñano et al., 2011; Hafez et al., 2011; Natarajan et al., 2011; Martin-Camean et al., 2014d). The MN assay produces a highly reliable, rapid and broad-spectrum determination of DNA damage at the chromosome level with limited costs and minimal time required to complete the test (Hvhannisyan et al., 2010; Natarajan et al., 2011).

Contradictory results from genotoxic tests have been reported in the scientific literature. These results depend on several factors: the specific appliance studied (mainly wires, bands, or brackets), appliance composition, appliance manufacturer, sample size (the number of orthodontic patients ranged from 15 to 55), time of treatment analysis (i.e., day of debonding, or shorter periods of 10 or 30 days, or 9 months), and genetic assays used (e.g., the comet assay or MN frequency). Furthermore, some studies compared the experimental group to an untreated group, while others evaluated genotoxic effects longitudinally in the same patients (Heravi et al., 2013).

The first *in vivo* study was performed by Faccioni et al (2003), who conducted the alkaline comet assay in 55 orthodontic patients after they received 2-4 years of conventional orthodontic treatment (e.g., bands, bonded brackets and archwires of different compositions). They reported genotoxic damage and found positive correlations between the concentrations of released Co and Ni and the number of comets as well as correlations between Co levels and comet tails. In contrast, Westphalen et al. (2008) found that 10 days after the placement of the orthodontic appliances, the comet assay results indicated these appliances (made of an SS alloy) did not induce any genetic damage. These differences may be explained by the analysed samples, the larger number of patients using the appliances for a much longer period (1.5-3 years), and the composition of the orthodontic appliances. In contrast, there was a significant increase in MN frequency 30 days after the beginning of the treatment. This investigation is an example of the benefits of combining two tests with supplementary characteristics (MN and comet assays) to obtain a better understanding of the potential genotoxic effects of orthodontic appliances. These discrepancies could be explained

because the researchers measured different specific genetic endpoints. Although the comet assay generally detects more DNA damage than the MN assay, the latter test was shown to be more sensitive in this study. Furthermore, the assays were applied at different time points, 10 d or 30 d for comet assay or MN, respectively, according to the type of DNA damage that was detected (i.e., primary and reparable effects in a short period of time for the comet assay and chromosomal damage at later stages).

According to Fernández-Miñano et al. (2011), genotoxicity induced in buccal cells could be related to the composition of orthodontic appliances. They used the comet assay to examine the effect of three different alloys (stainless steel, titanium and Ni-free) before the orthodontic treatment was applied and 30 days later. They concluded that the orthodontic apparatus made with Ti was not genotoxic for oral mucosa cells, whereas the SS alloy and Ni-free allow induced DNA damage in buccal mucosa cells. Significant release of Ti and Mn ions was detected in patients treated with SS tubes and brackets, and more Cr and Fe were detected in the Ni-free group. In contrast, Hafez et al. (2011) conducted a prospective longitudinal *in vivo* study with sixty patients over a 6-month period in which they observed that SS brackets with SS archwires produced the least biological damage, whereas titanium brackets with nickel-titanium archwires produced the greatest cytotoxicity and genotoxicity (according to the comet assay). The cellular ion content in buccal cell samples also varied over time. The Ni and Cr content increased progressively at 3 and 6 months compared to the content measure before treatment, which disagrees with previous studies that showed only a significant initial increase in metal content that decreases or stabilizes over time. Despite the bioaccumulation of these metal ions, Hafez et al., (2011) also reported damage to the DNA in mucosa cells at 3 months of treatment and not at 6 months. This result might imply recovery from the initial placement and the development tolerance or DNA repair. Therefore, regarding the treatment time points that were considered, the results are contradictory, with some authors indicating a recovery and others indicating increased damage.

Moreover, Natarajan et al., (2011) showed that the higher MN frequency induced by orthodontic appliances in oral mucosa cells reverted to normal values after only 30 days, and there was no correlation between the concentrations of released Ni and Cr ions and genotoxic damage. These results disagree with the previous studies mentioned above (Faccioni et al., 2003; Fernández-Miñano et al., 2011). In contrast with these results, Heravi et al., (2013) reported no significant differences in the frequency of MN cells before or 9 months after the appliance was placed. This result was confirmed in a study performed by Angelieri et al (2011) using the MN test. They found a lack of clastogenic and/or aneugenic effects from orthodontic appliances before, during, and after the therapy.

Fixed orthodontic treatment includes metallic components as well as orthodontic composites, and because the bonding agents are generally left in close contact with oral tissue over long periods of time, their biocompatibility is also a concern for orthodontic treatment. Thus, genotoxic studies have been conducted for the first time to evaluate the effects of three different light-cured orthodontic composites over a 6-month period. The researchers used the frequency of MN formation and nuclear alterations, such as karyorrhexis (KR), karyolysis (KL), and binucleated cells (BN) to evaluate biocompatibility (Toy et al., 2014). During the 6 months of orthodontic treatment, the composites did not show significant differences in MN rates within the same cell type. In contrast, morphological signs of cytotoxicity were observed, including an increase in the number of BNs for all the composite groups and a significant increase in the KL frequency between the beginning and the end of the study for both bonding composites. Similarly, the same authors employed these assays to evaluate the genotoxic effects of a banding procedure with 5 different orthodontic cements in oral buccal epithelium cells over a 1-month period (Öztürk et al., 2012). MN analysis revealed a significant increase in chromosomal damage between the beginning and 1 month time points for all of the tested orthodontic cements, and they also showed morphological changes. Further research is needed to confirm all these results and to assess the long-term biocompatibility and biological effects of these materials (i.e., composites for band cementation).

Recently, a study was carried out in our laboratory on human buccal cells by introducing some modifications to the comet assay. Our results revealed that conventional orthodontic appliances and the additional employment of miniscrews induced genetic damage compared to a control group (n=20), and the genotoxic effects of orthodontic miniscrews were similar to patients wearing traditional fixed orthodontic appliances even though applying miniscrews involves a more invasive procedure and closer contact with buccal cells (Martin-Camean et al., 2014d). The scarce research in this field, the importance of this subject for the health status of orthodontic patients and the wide use of miniscrews for distal or mesial movement of teeth, anterior retraction or protraction of teeth, intrusion or extrusion, indicate the need for further studies, with larger samples, and long-term follow-up analysis, about the use of miniscrews with different compositions.

4. Conclusions and future prospects

Experimental data show that the biocompatibility of orthodontic appliances depends on their composition and their corrosion behaviour, which can be improved by performing different techniques to reduce their deterioration. Due to the diversity in the composition of the materials and the manufacturing techniques applied to orthodontic materials, along with the variety of treatment lengths and intraoral conditions in orthodontics and the potential effects of these treatments on corrosion, safety evaluations of orthodontic materials on a case-by-case are required. Because a biocompatibility assessment is mandatory prior to their clinical use, the number of *in vivo* toxicity studies is small in the scientific literature compared to *in vitro* studies. However*, in* vivo studies provide valuable information on the effects of orthodontic materials in real clinical exposure scenarios. Therefore, further clinical studies (including studies of metal ion release, cytotoxicity, and genotoxicity), considering larger populations and longer treatment periods, and employing standardized methods, are necessary. Moreover, it is advisable to employ several genetic assays to evaluate genotoxicity. Additionally, *in vivo* monitoring studies of metal ions (such as Ni and Cr) are needed to investigate cause-effect relationships are required. These additional studies would allow

researchers to draw clear conclusions while also taking the contradictory results available now into account. Considering that metal ions can induce oxidative stress, further research is needed to determine whether the oxidative stress mechanism is involved in the toxic effects observed in the modified version of the comet assay that identifies oxidative genetic damage. Finally, it has been observed that most of the experimental studies have been performed with traditional orthodontic appliances (e.g., arches or bandwires), but other appliances that are more invasive, but widely used and relatively new (e.g., miniscrews), novel procedures and novel adhesion materials are also worthy of investigation.

Acknowledgements:

This work was supported by the Junta de Andalucía (Plan Andaluz Investigación, code CTS-358).

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table captions

Table 1. In *vitro* studies on the release of metals from orthodontic appliances: Experimental model and conditions, metal ions and concentrations released, and main results reported.

Table 2. *In vivo* studies on the release of metals from orthodontic appliances: Experimental conditions, biological matrix, metal ions and concentrations released, and main results reported.

Table 3. Cytotoxicity studies to assess the biocompatibility of orthodontic appliances: Experimental models and conditions, assays performed, metal ions investigated and main results reported.

Table 4. *In vitro* mutagenicity and *in vivo* genotoxicity studies performed in oral mucosa cells exposed to orthodontic appliances: experimental models and conditions, assays performed, metal ions investigated and main results reported.

Table 1

Table 1. In *vitro* studies on the release of metals from orthodontic appliances: Experimental model and conditions, metal ions and concentrations released, and main results reported

References	Orthodontic appliances	Experimental model	Solutions and incubation times, days	Metal ions investigated	Detection mode	Median or mean concentration of metal ions (µg/L or ug/g))	Main results
Suarez et al., 2010	Stainless steel (SS), Nickel-titanium (NiTi) and copper-nickel-titanium (CuNiTi) lingual orthodontic archwires	Saline solution	Samples were taken at 7, 14, and 30 days after immersion.	Ni	AAS	After 30 d accumulated Ni release (ng/mm$^{2)}$) oscillated between 0.001303 (SS D-Rect, the lowest) and 0.00362 (SS 0.016, the highest)	SS archwires released the highest amount of Ni. The amount of Ni released for all archwires is below the levels known to cause cell damage
Bhaskar and Subba Reddy, 2010	Bands, SS wires and space maintainers	Artificial saliva	37ºC. Samples were analyzed on 1,7,14,21, and 28 d	Cr, Ni	AAS	Nickel released ranging from 4.95 to 7.78 ppm, and C between 1.70 to 4.54 ppm	The peak of Ni and Cr released was on 7th day, and then the rate of release diminished
Sfondrini et al., 2010	3 kinds of brackets: new conventional SS, recycled SS, and Ni-free brackets	Artificial saliva at various acidities (pH4.2; 6.5 and 7.6)	After 15 min, 1 hour, and 24, 48 and 120 hours samples were analyzed	Ni	GFAAS and ICP-OES	Recycled brackets: 74.2 µg/g New SS brackets: 7.14 µg/g. Ni-free brackets: 0.03 µg/g	Reconditioned brackets released the most Ni, and the highest Ni release was performed at pH 4.2 and lower a t pH 6.5 and 7.6.In general, Ni release increased with the immersion time for all brackets
Ortiz et al., 2011	Stainless steel, nickel-free, and Titanium orthodontic alloys, tubes and brackets	Buccal tubes and 20 brackets were submerged in Minimal essential medium (MEM) supplemented with 0.5% fetal bovine serum	10 samples of the 3 different metallic materials submerged in MEM were taken after 30 days of incubation	Ti	ICP-MS	High concentrations of Ti, Cr, Mn, Co Ni, Mo Fe, Cu and Zn were detected.	The nickel-free alloy released lower amounts of ions to the medium.

Danaei et al., 2011	SS brackets kept in 3 different mouthwashes: Oral B, chlorhexidine, and Persica	Immersion in 3 mouthwashes	Immersion in the corresponding mouthwashes and deionized water , 37ºC for 45 d	Cr, Cu, Fe, Mn, Ni	ICP-OES	Cr, cu, Fe, Mn, Ni	The highest metals release was found with chlorhexidine compared with the other 2 mouthwashes
Espinar et al., 2011	NiTi archwires treated by oxidation for obtaining Ni-free surfaces	Artificial saliva	pH 7.4, 37ºC, for 30 days. Samples were taken at 1, 5 h, and at 1,2,5, and 15 days	Ni	GFAAS	---	The concentration of ion Ni initially increased very sharply and later reached a saturation level. The film of Titanium oxide reduced Ni release
Liu et al., 2011	Two kinds of commercial equiatomic NiTi archwires and half of the NiTi wires were exposed to continuous bending stress	Artificial saliva	8 groups (2 materials, 2 pH values (pH 2 and 5.3) and 2 loading conditions were investigated at 37ºC. NiTi wires were immersed and sampled at 1,3,7, and 14 days	Ni	ICP-MS	Under bending conditions Ni release rate ranged from 4.3 to 36.8 µg/cm^2 after 1 day of immersion	Bending stress induced greater Ni ion release compared with unstressed specimens in artificial saliva at both pH 2 and 5.3
Krishnan et al., 2011	Six groups of B titanium arch wires uncoated and PVC-coated	Artificial saliva and high fluoride ion concentration mouth rinse	Immersion cycle of 5 min three times a day., and also immersion in a commercial artificial saliva solution	Ti, Mo	ICP-AES	---No data	Mo release in negligible amounts, from wires immersed in fluoride mounthwash.
Reimann et al., 2012	New brackets and 4 types of recycled brackets (flame, acid bath, commercial unit Big Jane, and commercially recycled)	Artificial saliva	Static immersion for one week	Ni	ICP-MS	0.15 µg/day -0.18 µg/d depending on the treatment	No significant differences were observed in Ni release with the new brackets, those recycled by direct flaming and in acid bath
Gil et al., 2012	NiTi archwires heat treated at 500 and 600ºC for different	Artificial saliva	Archwires were immersed in artificial saliva at pH 7.4 and 37ºC	Ni	GFAAS		The Ni ion releases increased very fast initially , and after

	periods of time		for 30 days, and Ni was measured at hours 1 and 5, and at days 1, 2, 5 and 15.				reached a saturation level, The release of Ni was higher in the original archwires than in the reused ones, due to the formation of Ni-rich precipitates
Milheiro et al., 2012	5 different multi-stranded wires: 4 of them were stainless steel and 1 Ni-free	Distilled water of lactic acid	One group of wires were submersed in distilled water and another in lactic acid 90%, and each main group was divided into there subgroups: a) not exposed to cyclic loading; b) wires exposed to 1000 cyclic loading cycles , and c) exposed to 10000 cyclic loading. After 24 h, the wires were removed and samples were measured	Ni	ICP-MS	Non-loaded wires in water: 0.2-4.8 ppb; in lactic acid: 12.1-263.5 ppb. Wires 1000x cycles: in water: 1.1-9.3 ppb; in lactic acid: 14.8-263.4 ppb Wires 10000x cycles in water: 1.7-9 ppb; in lactic acid: 13.7-239.5 ppb	All wires released considerable amounts of Ni. Acidity has more impact in comparison to mechanical loading
Mikulewicz et al., 2012	Wires, brackets, bands and metal ligatures of stainless steel	Artificial saliva containing 0.40 g/L NaCl	Incubation for 30 days at 120 rpm, 36.6ºC	Al, Mg,, Si, P, S, K, Ca, Ti, V, Mn, Fe, Co, Cu, Zn, Ni and Cr	ICP-MS	Mg: 1,699; Al:559, Si:4.491; P:31,753; S:1,446,700; K 231,917; Ca:228,100; Ti 394: V:8; Mn:68; Fe 2,382; Co 4; Cu:121; Zn:91; Ni 573; Cr 101	Experimental group contained 39 times more Ni than de control solution. A correlation between Ni and Cr concentrations in saliva was found
Zhang et al., 2014	Composite arch-wires (CoAW)	Artificial saliva (AS), chloric solution (0.9% NaCl), fluorinated artificial saliva (0.1% NaF) and protein-containing artificial saliva (0.1% BSA)	4 different solutions	Cu	ICP-OES	Cu release ($\mu g/mm^2$) in simple artificial saliva:0.20; in chloric solution:0.56: in florinated –AS 0.40; in protein-containing AS:0.19	The CoAW samples were most susceptible to corrosion in the chlorine solution (greatest Cu release) and most resistant in the protein-

							containing artificial saliva
Sheibaninia 2014	NiTi orthodontic wires	4 groups: 2 immersed in artificial saliva at pH 7.4, and 2 groups immersed in artificial saliva+ascorbic acid to achieve pH 4.5. Each group was subdivided en wires not exposed to thermocycling and wires exposed to 500 thermocycling rounds	Solutions were incubated for 48 h at 37ºC. Thermocycling process was performed between 5-55ºC for 500 cycles	Ni	GFAAS	Mean Ni released per group: I (no treatment:)): 2.81 µg/L II (thermocycling): 6.11 III (pH 4.5): 3.99 IV (pH 4.5 and thermocycling): 9.74	Maximum Ni was released under acidic pH in the thermocycling group
Gonçalves et al., 2014	Stainless steel metallic orthodontic bands: Non-soldered bands (NSB) and silver soldered bands (SSB)	Bands were immersed in RPMI 1640 tissue-culture medium with 10%serum	Immersion for 24 h at 37ºC under agitation	Ag, Cd, Cu, Cr, Fe, NI, Zn	FAAS, GFAAS	NSB: Cr (ND); Fe (0.1 mg/L; Ni (0.05 mg/L) SSB: Cr (0.059 mg/L); Fe (0.619); Ni (0.629); Cu (20.35); Ag (1.381); Zn (5.276); Cd (0.004)	Ag, Cd, Cr, Cu and Zn were detected in SSB medium samples, and Fe and Ni were detected in both the SSB and NSB samples
Mikulewicz et al., 2014a	Wires, brackets and bands made of stainless steel, and elastic ligatures of a polymer	Thermostatic glass reactor with artificial saliva	Artificial saliva (0.5 mL/min) during 28 days	Ni, Cr, Cd, Cu, Fe, Mn, Mo, Si	ICP-OES	Total mass released during 4 weeks. Cu:31.3 µg Cr: 5.47 µg Ni:18.7 µg	The estimated doses of Cr, Cu and Ni released during the treatment were far below the toxic dose to humans.

* AAS: Atomic absorption spectrometry
FAAS: flame atomic absorption spectrometry
GFAAS : graphite furnace atomic absorption spectrometry
ICP-MS: Inductively coupled plasma-mass spectrometry
ICP-OES: Inductively coupled plasma-optical emission spectroscopy
PVC-coated: cathodic arc physical vapor deposition

Table 2

Table 2. In *vivo* studies on the release of metals from orthodontic appliances: Experimental conditions, biological matrix, metal ions and concentrations released, and main results reported.

References	Orthodontic appliances and time treatment (sample collection)	n	Matrix analyzed	Metal ions investigated	Detection mode	Median or mean metal concentrations (µg/L or ug/g))	Main results
Freitas et al., 2011	Sample collection: before placement of appliances, and 10 min, 24 h, and 7, 30, 60 days after placement	60 children: 30 control and 30 treated	Saliva	Ag, Cd, Cu, Zn	GFAAS	In the treated group, at 10 min after placement, the highest mean was for copper (70.60 µg/L), and the lowest for Zn (0.07 µg/L).	Great amounts of these ions ere released, with the highest concentrations immediately after placement of appliances
Mikulewicz et al., 2011c	Orthodontic appliances without specification	28 patients and 18 control subjects	Hair	Cr, Fe, Mn, Ni	ICO-OES	Control (µg/g dry hair mass) Cr: 0.1298; Fe:11.74; Mn:0.4850; Ni:0.3642 Patients (µg/g dry hair mass) Cr:0.1331; Fe:12.22; Mn:0.5739; Ni:0.5073	No significant differences in the content of metals in hair between exposed and non-exposed groups. The highest difference between groups was found for Ni (39%) and Mn (18%).
Amini et al., 2012	Stainless steel (SS) arches from different branches	28 patients and 28 control subjects	Saliva	Cr, Ni	AAS	Control: Cr: 2.6; Ni: 11.9 Patients Cr: 11.9; Ni:18.5	A statistical significant difference was found for Ni between both groups
Ousehal and Lazrak, 2012	Sample collection: before appliance placement, just after placement, and 8 weeks after placement.	16 orthodontic patients	Saliva	Ni			Significant increase in Ni levels just after NiTi archwire insertion. However, the difference was non-significant 8 weeks

							later
Amini et al., 2013	SS brackets, bands and NiTi archwires Sample collection at 4 times: Before treatment (T1); 3 months before induction stress (T2); 15 min after stress (T3) and 30 min after stress (T4)	30 orthodontic patients	Saliva	Cr, Ni	GFAAS	Cr: 11.9 µg/L to 14.1 µg/L Ni: 4. to 5.1 µg/L	Significant increase in Ni release into saliva at the stress induction, but Cr content was no significantly altered
Martin-Cameán et al., 2014a	SS alloys for brackets, tubes, bands and ligatures; NiTi and SS archwires	20 orthodontic patients and 20 controls	Oral mucosa cells	Ti, V, Zr	ICP-MS	Mean values for control group: Ti : 5.14 ng/g; Zr:<LOD Orthodontic patients: Ti: 5.23 ng/g; Zr: 0.54 ng/g	No significant differences were found in Ti concentrations in both groups
Martin-Cameán et al., 2014b	SS alloys for brackets, tubes, bands and ligatures; NiTi and SS archwires	20 orthodontic patients and 20 controls	Oral mucosa cells	Co, Cr, Cu, Ni	ICP-MS	Control group (median). Co: 0.6; Cr:2.3; Cu:4.9; Ni:4.3 Orthodontic group (median): Co:11.6; Cr:17.5; Cu:8.5; Ni:24.9	Significant higher values of all metals considered were detected in orthodontic patients in comparison to control group
Martin-Cameán et al., 2014c	SS alloy for brackets, tubes bands and ligatures NiTi alloy or SS archwires Treatment: At least 24 consecutive months	70 orthodontic patients and 56 controls	Hair	Cr, Cu, Fe, Mn, Ni	FAAS GFAAS	Median values for control group (µg/g dry hair mass): Cr:0.36;Cu:33; Fe:25.3; Mn:0.23; Ni:0.36 Median values for orthodontic group, Cr:0.33; Cu:24; Fe:24.86; Mn:0.42; Ni:0.33:	Only significant increased Mn levels in orthodontic patients was detected
Mikulewicz et al., 2014b	SS alloy for experimental plates, simulating orthodontic appliances. Sample collection: in the case of hair at 0, 3 and 6 months of treatment. Invasive samples at the end of experiment.(6 months)	12 pigs in the control group and 12 pigs received plates on the inner buccal side	Non invasive samples: Pig hair Invasive samples: kidneys, liver, lungs, aorta, oral mucosa from pigs	Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Si, Zn	ICP-OES	The highest differences in the content were found in aorta (Ni level was 4.8 times higher in the experimental group vs control), in the cheek (Ni 3.5 times higher) and in the hair after 3 moths (Cr 3.4 times higher).	Metals released have passed into selected tissues of pigs

Mikulewicz et al. 2015	SS alloy for brackets and ligatures NiTi for wires Sample collection: before and on 4^{th}, 8^{th} and 12^{th} months of the treatment	47 orthodontic patients	Human hair	Cr, Fe, Ni	ICP-OES	Mean values before treatment (mg/kg) Cr:0.0201; Fe:13.2; Ni:0.275 After 1 year treatment Cr:0.158; Fe:14.2; Ni:0.422	Only the content of Cr was statistically significantly higher during the treatment than before the beginning of therapy. After 1 year of treatment, an average accumulation of 8.94 µg Cr, 7.4 µg Ni and 131 µg Fe was found,

FAAS: Flame-atomic absorption spectrometry
GFAAS: Graphite furnace atomic absorption spectrometry
ICP-OES: Inductively coupled plasma-optical emission spectroscopy
LOD: Limit of detection

Table 3

Table 3. Cytotoxicity studies to assess the biocompatibility of orthodontic appliances: Experimental models and conditions, assays performed, metal ions investigated and main results reported.

References	Experimental model	Assays performed	Orthodontic appliances	Solutions and incubation times	Metal ions investigated	Metal concentrations	Main results
Assad et al., 1994	L-929 fibroblasts	Proliferation by light microscopy	NiTi, Ti, vitallium (Co-Cr-Mo) and 316L SS discs	Cells were exposed directly or under an agar bed	None	--	Decreasing order of cytotoxicity: NiTi ~ Co-Cr-Mo >> pure grade 4 Ti ~ pure grade 1 Ti ~Ti 6A1 4V ~316L stainless steel.
Wever et al., 1997	Human skin fibroblasts (PK84)	Dilution minimal essential medium extract test	Ni-Ti alloy for wires AISI 316 LVM Control -: UHMW polyethylene Control +: RIVM-pos. Latex	100-12.5% extracts diluted in minimal essential medium (MEM) , at 24, 48 and 72 h	None	--	No cellular lysis, intracellular granulation or morphological changes were found
Ryhänen et al., 1997	Human osteoblasts and fibroblasts	Cell proliferation	NiTi alloy (Nitinol), stainless steel (SS) titanium (Ti), composite material (C)	Exposure to disc materials for 10 days	Ni and Ti by GFAAS	Nitinol released more Ni than SS but after 2 days concentrations were about equal	Nitinol has good in vitro biocompatibility
Rose et al., 1998	L-929 mouse fibroblasts	MTT test	23 different orthodontic wires made from 5 different alloys	Cells were exposed for 72h to eluates of the materials	Ni, Fe, Mn, Cr, Co, Zn, Mob by ICP-AES	Different degree of ions released depending on the material	The NiTi wires Nitinol, Sentalloy and Original Chinese Wire and the β-titanium alloy TMA had no effect on the rate of cell proliferation. Nor did stainless steel wires inhibit growth significantly, with the exception of Australian Wire and Wildcat Wire. The manganese-steel alloys Noninium and Mezanium caused significant reductions in growth rate. The most severe growth inhibition was caused by the Co-Cr-Ni alloy Elgiloy. In terms of the qualitative ISO assessment all the test samples investigated corresponded to a cytotoxicity grade of 0, i. e. non-cytotoxic.

Tomakidi et al., 2000	Immortalized human gingival keratinocyes (IHGK)	Neutral red staining Trypan blue staining Propidium iodide staining Hexosaminidase assay	Ni-free wire; an UK-1 bond, Ni-free and Ni-containing brackets ; Titanium expansion screw	Lactic acid: NaCl (0.1M) 50:50 1,3,7, and 14 days	Cr, Fe, Ni by AAS	Fe>Cr>Ni. Fe and Cr release decreased from day 1 to 14. Ni showed a different pattern	Lack of damage of cell membranes was confirmed by the results obtained from the qualitative staining techniques (neutral red/trypan blue and propidium iodide assays) and using the hexosaminidase assay
Es-Souni et al., 2001	Cultured epithelial cells from explants of human gingiva	MTT tests and morphological observations by SEM	Binary NiTi42 and a ternary NiTi42Cu7 alloy	Cells were exposed to the alloys for 24, 48 and 72h	Cu by AAS in the ternary alloy	Cu is released after an incubation period of 7 days at 37ºC.	Cytotoxicity of the ternary alloy is higher than that of the binary alloy. This is related to the release of Cu.
Es-Souni et al., 2002	Fibroblast cells from explants of human gingiva	MTT test	Two arch wires: - Neosent alloy F 80 (Ni57.6Ti42.4 wt%) - SE NiTi (Ni57.8Ti42.2 wt%)	24, 48, 72h	None	--	Neosent is better tolerated than SeNiTi. The survival rate of the cells cultured with Neosent is ~90.75% and with SeNiTi between 77 and 85%. Since the chemical composition is the same, the net difference in the biocompatibility is thought to arise from the difference in the corrosion behavior
Mockers et al., 2002	Mouse fibroblasts L929	MTT assay	28 new and 9 clinically used materials, including brackets, molar bands and archwires. The metallic materials were made of SS, gold-plated steel, pure titanium, NiTi, Ti-Mo and silver-based soldering alloy. The non-metallic materials were in polycarbonates and ceramics	Release period of the material in the culture medium (0.1 mg/mL) for 3 and 14 days	None	--	The metallic and non-metallic materials were similar in terms of cytotoxicity. The cytotoxicity of clinically used samples was equivalent to that of the non-used samples, except a cytotoxic sample, at 14 days, corresponding to a soldered and clinically used molar band. The 3 days results were different from the 14 days results in 6 cases out of 37. The materials can be considered as non cytotoxic

El Medewar et al., 2002	Human epithelial embryonic cells (L132) Human Embryonic Palatal Mesenchymal cells (HEPM)	Colony forming method on L132 cells Cytoskeletal organization in HEPM cells	NiTiNOL, highly pure Nickel (hp-Ni) and commercially pure Ti (cp-Ti)	Cells were exposed to disks of each alloy for 72h	Ni and Ti by ICP-AES in the culture medium	Ni-release is very low from the NiTiNOL but rather high from hp-Ni samples. Ti release is very low from cp-Ti and increased slighty from NiTiNOL	hp-Ni is a cytotoxic material. Nevertheless, its toxic potency depends on the cell type, it is more toxic for L132 cells than for HEPM cells. cp-Ti and NiTiNOL are biocompatible.
Assad et al., 2002	L-929 mouse fibroblast cells	Elution test Morphology by microscopy Cell layer reactivity	Porous titanium–nickel (PTN) alloys Control -: polyethylene Control +: phenol	Cells exposed to extracts	None	--	Porous titanium–nickel can be considered completely cytocompatible
Bogdanski et al., 2002	Osteoblast-like osteosarcoma cells (SAOS-2, MG-63), primary human osteoblasts (HOB), and murine fibroblasts (3T3)	Cell adhesion and proliferation by light microscopy	Functionally graded material: Ten mixtures from Ni:Ti=90:10 to Ni:Ti=10:90 and pure Ti	Cells were exposed directly to the material 3-4 days	None	--	NiTi alloys do not have cytotoxic effects up to a Ni content of 50%.
Faccioni et al., 2003	Epithelial cells of buccal mucosa from each patient	Trypan blue exclusion test	55 orthodontic patients with fixed appliances in both arches. The fixed appliances consisted of an average of 4 to 8 bands and 20 bonded brackets; the material was American Iron and Steel Institute (AISI) type 304 for the bands and type 316 for the brackets. The archwires were NITi alloy, SS or chromium-cobalt-nickel alloy. 30 control subjects without orthodontic treatment	Non-specified.	Co and Ni by ICP-MS	Co and Ni concentrations were about 2.8-fold and 3.4-fold higher, respectively, than those in the control subjects	Significant decrease in cellular viability (%), 73.43 ±12.29 and 50.40 ± 13.55 in controls and patients, respectively

Eliades et al., 2004	Human periodontal ligament fibroblasts and gingival fibroblasts	Cytotoxic effects: MTT test Cytostatic activity: DNA synthesis assays	SS brackets and 0.018 0.025 Ni-Ti archwires	Saline solution for a month Nickel chloride as a positive control	Ni and Cr by ICP-AES	Measurable Ni and traces of Cr were detected only for the SS group, but no release was identified for the Ni-Ti wires.	Concentrations of the nickel chloride solution > 2 mM reduced by more than 50% the viability and DNA synthesis of fibroblasts; however, neither orthodontic materials-derived media had any effect on the survival and DNA synthesis of either cells
Hanson and Lobner, 2004	Murine cerebral cortical cell cultures	Release of the cytosolic enzyme lactate dehydrogenase (LDH)	3 latex and 3 nonlatex orthodontic elastics	24h after placing the piece of elastic in the bathing media	None	--	The nonlatex elastics did not cause significant neuronal death, but exposure to each of the latex elastics resulted in significant neuronal death. The neuronal death induced by each of the latex elastics was blocked by adding a metal chelator. This suggests that the cause of the latex cytotoxicity was zinc release
David and Lobner, 2004	Murine cerebral cortical cell cultures	Release of the cytosolic enzyme LDH Propidium iodide staining	NiTi, Cu-Ni-Ti, Ti-Mo, Elgiloy and SS archwires alloys	Samples were place on tissue culture inserts suspended above the cell cultures for 24h	None	--	NiTi, Cu-Ni-Ti, Ti-Mo were not neurotoxic, while Elgiloy and stainless steel were significantly toxic. The death was free radical mediated. SS induced apoptosis
Oh and Kim, 2005	L-929 cells, a mouse fibroblast cell-line	Neutral red staining	Four types of SS wires These wires were heat-treated in a vacuum, air, or argon environment, and were cooled in either a furnace or a water bath. Control +: Cu alloy. Control -: Polyethylene	Artificial saliva 24h	Ni by GF-AAS	In all groups, the dissolved Ni ions in artificial saliva was lowest for the vacuum heat treatment-furnace cooling group and a significant difference was shown compared with the other experimental groups.	The cytotoxicity was mild in all the experimental groups but the response index of the air groups was slightly higher than in the other groups
Nocca et al., 2006	Mouse fibroblast cell line (3T3 Swiss)	MTT assay	Self-curing and a light-curing orthodontic composite resins	24h extracts method and indirect toxicity method	None	--	The chemical-cured material is more cytotoxic than the light-cured one
Kao et al., 2007	Human osteosarcoma cell line (U2OS)	MTT assay	Fluoride corrosion extracts of stainless steel (SS) and nickel-	The SS and NiTi wires were corroded with the application of 3	Ni, Cr, Ti by AAS	The release of ionic Ni showed statistically significant differences	Only orthodontic wires in acidulated fluoride saliva solution can cause U2OS cell toxicity

			titanium (NiTi) wires	kinds of electrolytes: 0.2% pH 3.5 acidulated phosphate fluoride (NaF) in artificial saliva, and pH 4 and pH 6.75 artificial saliva solutions. Cells were exposed for 24h.		in all groups	
Kobayashi et al., 2007	Squamous carcinoma cells	Cell growth Morphology by SEM	Diamond-like carbon (DLC) film coated and non coated NiTi orthodontic archwires	Materials were immersed in each well with cells directly for 96h	Ni by MIP-MS	Ni ions released in the solution was reduced by one-sixth using DLC coating compared with non-coated wire.	DLC film-coated wires showed higher cell growth rate in comparison with the non-coated wires. From SEM observation, there were no differences between DLC coated and normal NiTi wires
Malkoc et al., 2010	L929 cells	MTT assay	5 different light-cured orthodontic bonding composites	Eluates of the composites in medium. 24h of exposure	None	--	Only Transbond XT showed significant cytotoxicity compared with the control group
Ortiz et al., 2011	Human fibroblast cell line 142BR	MTT assay	3 different metallic materials (buccal tubes and brackets): stainless steel, Ti and Ni-free	Materials were submerged for 30 days in MEM. Cells were exposed for 7 days.	Ti, Cr, Mn, Co, Ni, Mo, Fe, Cu, Zn, As, Se, Cd, Pb	In the SS extract 7 metals were found in greater concentration (Ti, Cr, Mn, Co, Ni, Mo, Fe)	Stainless steel was the material that induced a higher decrease in cell viability (4.60%)
Hafez et al., 2011	Buccal mucosa cells *in vivo*	Trypan blue exclusion dye test	40 patients with orthodontic appliances and 20 subjects as control. The appliances consisted of 4 bands, brackets and archwires	Before orthodontic treatment and 3 and 6 months after appliance placement	Cr and Ni by GF-AAS	Ni and Cr increased with time	Cellular viability decreased but this was not evident at 6 months, possibly indicating tolerance for or repair of the cells. Stainless steel brackets and archwires showed the least biologic damage, whereas Ti brackets and archwires produced the greatest cytotoxicity
Angelieri et al., 2011a	Exfoliated buccal mucosa cells from adults (*in vivo*)	cellular death (pyknosis, karyolysis, and karyorrhexis)	23 healthy adults undergoing orthodontic therapy. The fixed appliances consisted of an average of 4 to 8 bands and 20 bonded brackets. The arch wires used were a NiTi alloy during the	Cells were collected before, during orthodontic therapy (170 days after beginning the treatment), and after therapy (ie, at least 6 months after the insertion of SS arches)	None	--	Orthodontic therapy was not able to increase other nuclear alterations closely related to cytotoxicity such as karyorrhexis, pyknosis and karyolysis

			treatment or SS at the end of the orthodontic therapy				
Spalj et al., 2012	Mouse fibroblast cells L929	Trypan blue dye exclusion test	Six types of orthodontic archwires were tested: (1) SS, (2) NiTi, (3) copper-nickel-titanium, (4) rhodium-coated nickel-titanium, (5) cobalt-chromium Blue Elgiloy, and (6) Ti-Mo	Cells were exposed to artificial saliva eluates for 48h	None	--	SS archwires have the highest and nickel-titanium the lowest biocompatibility
Zhang et al., 2014	Murine L-929 cells	MTT assay	Composite arch-wire (CoAW)	Cells were co-cultured with CoAW extract for 1, 2 and 4 days	Cu by ICP-OES	Cu ions released was lower than average daily dietary intake levels.	The viability of cells cultured in CoAW extract solution remained greater than 80% over 4 days of culture, indicating that the CoAW extract solution was not cytotoxic
Gonçalves et al., 2014	HepG2 cells and Human oral keratinocyte (HOK) cell line	MMT assay	SS orthodontic bands: Non-soldered bands (NSB) and silver soldered bands (SSB)	MTT assay: 3 and 24 h of exposure to NSB, SSB or negative control culture medium HOK cells: 24 h	Ag, Cd, Cu, Cr, Fe, Ni, Zn by AAS	Ag, Cd, Cr, Cu and Zn were detected in SSB medium samples, and Fe and Ni were detected in both the SSB and NSB medium samples	The SSB bands induced stronger cytotoxicity than NSB group in both cell lines
Toy et al., 2014	Exfoliated buccal ephitelial cells	Microscopy observation	Three different light-cured orthodontic bonding composites	Before the treatment, and at 1, 3 and 6 months after treatment cells were scraped	None	--	Some morphological evidence of cytotoxicity was seen for the 6-months orthodontic treatment
Bueno & Basting, 2014	Human osteoblasts	Proliferation by Tripan blue exclusion method Morphology by SEM	Two mini-implants (Morelli and Neodent) Control -: polystyrene	Osteoblasts were cultured on the surface of mini-implants for 24, 48, 72h	X-ray fluorescence spectrophotometry	Ti>Al>V>Fe	Osteoblast proliferation was successful, which increased over time without a significant difference between commercial brands

AAS: Atomic absorption spectrometry
GFAAS: Graphite furnace atomic absorption spectrophotometry
ICP-OES: Inductively coupled plasma-optical emission spectrometry
MIP-MS: microwave induced plasma mass spectrometry
MTT : 3(4,5-dymethylthiazole-2-yl)2,5-biphenyl tetrazolium bromide
SEM: Scanning Electron Microscopy
SS: Stainless steel

Table 4

Table 4. *In vitro* mutagenicity and *in vivo* genotoxicity studies performed in oral mucosa cells exposed to orthodontic appliances: experimental models and conditions, assays performed, metal ions investigated and main results reported.

References	Experimental models	Assays performed	Orthodontic appliances and experimental exposure conditions: solutions and incubation times (*in vitro*) and time treatment (*in vivo*)	Metal ions investigated	Metal concentrations found in cell lines (*in vitro*) or in buccal mucosa cells (*in vivo* studies)	Main results
Wever et al., 1997	*Salmonella typhimurium* strains (TA1535, TA 100, TA 1537, TA 98) with and without S9 Chinese hamster fibroblast cell line V79	Mutagenicity: Ames test Chromosomal aberration test (CA)	Ni-Ti alloy for wires 100-12.5% extracts diluted in minimal essential medium (MEM , at 24, 48 and 72 h	Not specified	None	No mutagenic effects were found with and without metabolic activation No significant increases in CA was observed
Tomakidi et al., 2000	Comet Assay	Immortalized human gingival keratinocytes (IHGK)	Ni-free wire; 3 tupes of brackets: an UK-1 bond, Ni-free and Ni-containing brackets; and also 1 Titanium expansion screw Solutions: Lactic acid: NaCl (0.1M) 50:50, and test materials were treated at 37ºC for several incubation times: 1,3,7, and 14 d	Cr, Fe, Ni measured by AAS	For all materials, the release of Fe, the most abundant corrosion product, ranged between 0.90 µg/cm^2 (Ni-free) to 123 µg/cm^2 (in brackets). Cr values ranged between 0.02-35 µg/cm^2 Values of Ni ranged from 0.02 (Ni-free) to 18 (brackets) µg/cm^2	Metal ions were released in this decreased order: Fe> Cr>Ni. None of the test eluates had any genotoxic effects (tail moment)

Faccioni et al., 2003	55 orthodontic patients and 30 control subjects	Alkaline comet assay	Bands (type 304), bonded brackets (type 316) and archwires of Ni-Ti alloy, stainless steel (SS) or Cr-Co-Ni alloy	Co, Ni by ICP-MS	Co and Ni were significantly higher in orthodontic patients (2.8-fold and 3.4 fold) in comparison to control subjetcs	Higher frequencies of cells with comets and apoptosis were found in the patient group. Positive correlations between Co and Ni--number of comets were found, and Co levels and comet tails
Montanaro et al., 2005		Mutagenicity : Ames test Sister chromatid exchanges (SCE) CA	Ni-free stainless steel (P558) and the conventional stainless steel AISI 316 L	None		The Ames test showed that the extracts of both materials are not mutagenic Both materials did not cause increases in the average number of SCE an in CA
Westphalen et al., 2008	20 orthodontic patients	Micronucleus (MN) and Comet assays	SS alloy Samples were obtained: 1) before and 30d after placement of the orthodontic appliances for MN assay, and 2) before and 10 d after treatment for the comet assay	None	None	The MN assay showed increase in MN cells 30 d after the treatment The comet assay results reveal that the treatment did not induce any genetic damage
Fernández-Miñano et al., 2011	15 orthodontic patients and 15 control subjects	Comet assay	Tubes and brackets of stainless steel, titanium and nickel-free Samples were taken before the treatment	Co, Cr, Fe, Mn, Mo, Ni, Ti by ICP-MS	Co, Mo and Ni were not detected Cells in contact with stainless steel alloy displayed the highest Mn and Ti concentrations Cells in contact with the Ni-free ally presented highest Fe and Cr ions	Stainless steel and nickel-free materials induced a higher DNA damage than the Ti alloy
Natarajan et al., 2011	20 orthodontic patients and 20 untreated subjects	MN assay	Samples were collected at debonding and 30 d after debonding	Cr, Ni by ICP-MS	Cr and Ni ion contents were no significantly different between experimental an control groups	MN frequency was higher in the treated group at debonding, whereas no significant differences 30 d after debonding were found.

Hafez et al., 2011	28 orthodontic patients and 18 control subjects	Comet assay	Buccal mucosa cells Collection samples: before treatment, and 3 and 6 months after appliance placement	Cr, Ni by GF-AAS	Cr and Ni increased after 6 months of treatment..	Fixed orthodontic appliances induced DNA damage, but these changes were not evident at 6 months. When compared with the control group, the changes were significantly only for cellular Cr content and DNA damage at 3 months,
Angelieri et al., 2011a	23 patients	MN assay	The fixed appliances consisted of an average of 4 to 8 bands and 20 bonded brackets. The arch wires used were a NiTi alloy during the treatment or SS at the end of the orthodontic therapy. Cells were collected before, during orthodontic therapy (170 days after beginning the treatment), and after therapy (ie, at least 6 months after the insertion of SS arches)	None		MN frequencies were not significantly different before, during, and even after orthodontic therapy
Angelieri et al., 2011b	Chinese hamster ovary (CHO) cells	Comet assay	Four different commercial orthodontic brackets wit similar chemical composition Brackets submersed in acetic acid and NaCl 0.1 M for different times: 1, 3, 7, 14, 21, 35 and 70 days. Cells were added to eluates maintained for 30 min at 37ºC.	None	None	The corrosion eluates did not induce genetic damage
Öztürk et al., 2012	55 patients	MN assay	Patients were divided in 5 groups, according to the orthodontic cement assayed 1 month period	None	None	A significant increase in chromosomal damage in all groups was reported. Band cementation with conventional glass ionomer cement showed the least genotoxic effects

Heravi et al., 2013	25 orthodontic patients	MN assay	Stainless steel brackets and Ni-Ti or stainless steel archwires Samples were taken before the appliance placement and 9 months later	None	None	No significant differences were found in the MN counts before and 9 months after therapy.
Toy et al., 2014	30 orthodontic patients	MN assay	Brackets and molar tubes were bonded with three different light-cured bonding composites Samples were taken before and at 1,3,6 months after treatment	None	None	MN rates did not significantly differ within the dame cell type, so, genotoxic effects were absent
Martin-Cameán et al., 2014d	20 controls, 20 orthodontic patients and 20 orthodontic+mini-implants	Comet assay		None	None	
Gonçalves et al., 2014	Comet assay Modified comet assay: FPG and Endo III enzymes* CBMN-Cyt assay*	HepG2* cells and Human oral keratinocyte (HOK) cell line	Ss metallic orthodontic bands: Non-soldered bands (NSB) and silver soldered bands (SSB) HOK cells: 24 h	Ag, Cd, Cu, Cr, Fe, Ni, Zn by AAS	Ag, Cd, Cr, Cu and Zn were detected in SSB medium samples, and Fe and Ni were detected in both the SSB and NSB medium samples	- NSB and SSB induced genotoxicity in comet assay, and stronger effects were found in the SSB group. - Both groups induced similar increases in oxidative DNA lesions - Nucleoplasmic bridges, biomarkers of DNA misrepair and/or telomere end fusions were elevated in the SSB group.

AAS: Atomic absorption spectrometry
CBMN-CYt: cytokinesis-bloc micronucleus cytome assay
FPG: Formamido pyrimidine glycosylase protein; Endo III: Endonuclease III
HepG-2: human hepatocellular carcinoma cell line
ICP-MS: Inductively coupled plasma-mass spectrometry

CAPÍTULO 7 / CHAPTER 7

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GENOTOXIC, CYTOTOXIC EFFECTS AND GENE EXPRESSION CHANGES INDUCED BY ORTHODONTIC FIXED APPLIANCES OVER ORAL MUCOSA CELLS: A SYSTEMATIC REVIEW.

Dental Materials (en revisión) **2015**

Genotoxic, cytotoxic effects and gene expression changes induced by orthodontic fixed appliances over oral mucosa cells: A systematic review

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ABSTRACT

Genotoxic, cytotoxic effects and gene expression changes induced by orthodontic fixed appliances over oral mucosa cells: A systematic review

Background: Accumulated chronic or severe acute DNA and cellular damage over oral mucosal cells constitutes one of the main initiating factors contributing to a wide range of malignant lesions to occur in the oral cavity. Eventually, oral mucosa remains to be persistently disrupted by intraoral appliances used in dentistry. Specifically, the types of fixed appliances used in orthodontics exert a maintained and localized aggression to the oral mucosa for a substantial period of time. Nevertheless, to date, notable controversy is found in literature regarding the effect of such sustained damage to the oral mucosal cells at genotoxic and cytotoxic level. The present systematic review aimed to investigate the effects of these interventions and is reported in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. ***Material and Methods:*** Electronic [CENTRAL,MedLine;SCOPUS; EMBASE, Cochrane Library, ISI Web of Science, PASCAL, OVID HealthSTAR, and EBM] and manual searches [OpenGrey; Google Scholar] up to 29th April, 2015 were conducted for publications of RCTs/quassiRCTs analyzing the genotoxic/cytotoxic effects of these types of oral appliances in humans. A primary outcome (cellular and DNA damage) and a number of secondary outcomes were examined. Two reviewers performed the study selection and the risk of bias assessment [Cochrane Collaboration's tool] and meta-analysis was conducted when possible on homogenous groups. ***Results:*** From the initial electronic search (27902), 17 articles were retrieved and 6 studies [low risk of bias(LRB)] finally met the eligibility criteria [*PICOS*]. Despite substantial heterogeneity in the methods and sampling, in most comparisons made, significant differences were observed by most of the studies (5 out of 6) regarding a critically acute detectable geno and cytotoxic effects after appliance using, in the short (at 1 and 3 months) and long term (24-48 months) evaluation. Nevertheless, some of the studies evaluating post-removable effects (2 out of 3) conclude that this effects at the DNA or cellular level were not statistically significant different to controls after removing the oral aggresion ***Conclusions:*** Acute DNA and cellular damage over oral mucosa cells are found induced by direct contact of orthodontic appliances as reported by sound selected scientific literature (LRB).Nevertheless, despite no further detection of these effects is described by a few studies after removing the appliances, additional rigorous randomized clinical trials are needed to explore to what extent no acquired damage is observed in the oral mucosa.

Keywords: Systamatic Review, Metaanalysis, orthodontic appliance, DNA damage, Genotoxicity, Cytotoxicity, oral mucosa, metal release

1. Introduction

Dental materials ought to guarantee absolute safety, biocompatibility and health compatibility in the short and long term use [1]. These qualities are of dramatic importance in the oral cavity medium where a hostile chemical microenvironment and high standards of mechanical resistance requirements are usually found [2]. Eventually, oral mucosa remains to be persistently disrupted by intraoral appliances used in dentistry. Specifically, the types of fixed appliances used in orthodontics exert a maintained and localized aggression to the oral mucosa for a substantial period of time [3]. To this respect, accumulated chronic or severe acute DNA and cellular damage over oral mucosal cells constitutes one of the main initiating factors contributing to a wide range of malignant lesions to occur in the oral cavity [4]. Not only this but also chemical/mechanical corrosion and metallic ions releasing [Nickel, Chromium, Titanium, Cobalt among many others], is a well-documented intraoral event that occurs in this type of appliances [5]. Various studies have evaluated the discharge of metal ions from orthodontic appliances in biologic fluids, and most have concluded that they do not reach toxic concentrations [6,7]. However, it cannot be excluded that even nontoxic concentrations might be sufficient to produce biologic changes in the oral mucosa. Despite the low release of ions from metal appliances, these can be taken up by the adjacent oral tissues [8-10] over the long period of orthodontic treatment and may possibly lead to genome alteration in the oral tissues of patients wearing them. Metals are not biodegradable, and their sustained release might produce irreversible toxic effects from their accumulation in the tissues [11]. To this regard, it´s been suggested that direct metallic ion exposure to Cobalt or Nickel is associated with several types of cancer in other mucosa [12].

Nevertheless, notable controversy is found to date in literature regarding the effect of such sustained damage to the oral mucosal cells at genotoxic and cytotoxic level. The present review aimed to systematically make a critical and analytical report of the available scientific evidence about the genotoxic and cytotoxic effects derived from of the short and long term metallic exposure over oral mucosa cells reporting in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [13].

2. Materials and Methods

Protocol and Registration

This systematic review was conducted is reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [13] and following the Cochrane Collaboration [14] and the Centre for Reviews and Dissemination guidance [15]. Neither a review protocol was available nor was the review registered.

Information resources and search strategy (Figure 1. Appendix Table1)

The databases explored were the CENTRAL, MEDLINE Database (Entrez PubMed, www.ncbi.nim.nih.gov), SCOPUS (www.scopus.com), EMBASE and The Cochrane Library (www.thecochranelibrary.com), ISI Web of Science, PASCAL, OVID HealthSTAR, and EBM (Evidence-Based Medicine) Reviews comprising of the Cochrane Central Register of Controlled Trials, Cochrane Database of Systematic Reviews (CDSR) and Database of Abstracts of Reviews of Effects (DARE). We included all articles published up to 29 April 2015. No restrictions regarding language or publication year was set. In addition, the OpenGrey database in EAGLE (European Association for Grey Literature Exploitation) and Google Scholar was searched for grey literature. Other sources and journals highly likely to contain studies relevant to the reviewed topic were manually searched also up to 2 May 2015. The present research was based on the MesH terms and combinations compiled in *Appendix*Table 1 for every database and all duplicates were electronically removed. When additional information was needed, efforts were made to contact the authors.

Eligibility / Criteria for Studies to be Considered (PICOS)

- Participants: patients undergoing orthodontic treatment with fixed appliances.
- Interventions: Assessment of DNA damage, genotoxicity and cytotoxicity on oral mucosal cells.

• Comparators/Control: internal or external control group with no orthodontic treatment, or placebo.

• Outcomes:

(1) Primary outcomes: quantification of DNA damage, genotoxicity and cytotoxicity in cells obtained from oral mucosa.

(2) Secondary outcomes: additional quantification of oral mucosa cells´ damage by including ion concentration or changes in gene expression by PCR, array or other biomolecular techniques.

• Studies: Controlled randomized and *quasi* randomized clinical trials (RCTs and q-RCTs).

Other types of article that did not match the targets of this review were not included in the final selection. Studies regarding removable orthodontic appliances, acrylic-based materials, descriptive studies, case reports, case series, opinion articles, reviews, animal and in vitro studies were excluded.

Data Collection and Analysis

Study selection

Two reviewers independently selected eligible studies by reviewing the list of titles/abstracts according to the inclusion/exclusion criteria. From eligible titles/abstracts the full articles were retrieved and the same reviewer independently reviewed the full articles to establish eligibility. Any discrepancies between reviewers for inclusion of articles were addressed through discussion until consensus was reached. Studies excluded at this stage were identified and reasons for exclusion recorded.

Data collection process

Based on Cochrane recommendations a pre-piloted standardized extraction form was used. Eligible studies' data were extracted and recorded by the first reviewer. The second reviewer crosschecked the accuracy and validity of all data extracted. In this

stage, authors of selected studies where directly contacted when necessary if any missed data was found in the paper.

Quality evaluation and Risk-of-Bias Assessment

Quality of the methods used in the included studies was assessed by two reviewers by using the Cochrane risk of bias tool [14]. Briefly, each of the keypoints in the tool was rated and a risk of bias evaluation allowed classifying the included studies as low, unclear, or high risk of bias. Sample size calculation was also examined.

Analysis of the numerical data

Data analysis for the present systematic review followed Cochrane guidelines for statistics with RevMan, 2012(version 5.2) [14] that allow us to make the comparisons of the effects observed from the different exposures to metallic ions. Mean differences with a 95% confidence interval was used for continuous data by using the Epidat software (3.0 version).

A meta-analysis was planned if relative homogeneity were observed between selected studies and if the outcome measurements warranted a meaningful statistical combination. Original outcome data, if possible, were subjected to meta-analysis statistical pooling was calculated by using Epidat 3.1 (Consellería de Sanidade, OPS-OMS, Spain). Mean difference (MD) was used for statistical pooling for continuous data and standard error of the mean. We computed the random-effects method, based on the assumption that variability in the retrieved studies could influence the effects being investigated, as described by Borenstein et al. [16] Heterogeneity and inconsistency across studies was assessed through the I2 statistic and DerSimonian and Laird's Q statistic [17], respectively with a value greater than 50 per cent being considered substantial heterogeneity. Finally, Egger's test [18] and Begg's test and funnel plot [19] were used to evaluate publication bias. Furthermore, sensitivity analysis was conducted to assess the robustness of meta-analysis. A p-value of < 0.05 was considered significant.

3. Results

Search

The search strategy identified a total of 27902 studies from all databases explored. After pre-selection the full texts of 17 potentially eligible papers were retrieved and examined fully and final selection included 6 studies that met all required criteria. More detailed explanation of the selection screening process is compiled in Figure 1.

Description of Studies

Six studies finally met the selection criteria for inclusion [8,20-24]. The main characteristics of eligible studies are summarized in Table 2. The excluded papers and criteria used for exclusion are available upon request.

Quality evaluation and Risk-of-Bias Assessment

None of the included studies was at low risk of bias across all keypoints/domains (*Appendix* Figure 2-3.). All researches were not assessed as low risk of bias because fail of reporting information in the study or after contacting the authors or because none or the reviewers could make a clear assessment in at least one of the keypoints/domains of the Cochrane risk for bias assessment tool. Of the six studies that met full criteria for final inclusion, none of them described a previous sample size calculation, detecting some concerns regarding inadequate statistical power (< 80%) for some of them.

Effects of interventions (metal exposure) over oral mucosa

Quantification of DNA and cellular damage: gene expression changes, genotoxic, cytotoxic effects.

The reviewed researches varied widely in the time point of exposure before sample collection and assessment of DNA / cellular damage. For the purpose of this review, the interventions were classified into 3 groups, based on the length of metallic appliance exposure against the oral mucosa: (1) short term assessment [<9 months metal exposure]; (2) long term assessment [>9 months metal exposure]; (3)

posttreatment assessment [sample collection after up to 18 months of metal exposure and after removing of the fixed appliance].

Three comparisons were made among the effects of the different interventions. Data obtained from the statistical analysis between-groups comparisons are described along the text, with the primary outcomes results described at short, long-term and post-treatment follow-up time points compiled in the *Appendix*Figure 4-6. Data on all secondary outcomes are available upon request.

(1) Short term assessment [<9 months]

Comparison of treated subjects group 1 (short-term assessment) to untreated controls was analyzed by several authors [20,22] with the main difference being the fixed appliances (tubes, braces and archwires) in direct contact to mucosa for less than 9 months. To this respect, one study [20] showed that after 3 months the composite score calculated for DNA damage value decreased to 108.4 ($p>.05$) and then normalized to 98.8 ($p<.05$) after 6 months of direct contact with the fixed metallic appliances compared to values of 125.6 observed for internal pre-exposed controls. But not only ion liberation by the whole metallic fixed appliances induces this type of results. When evaluating results from the effect of passive (no archwires) metallic appliances (stainless steel and nickel free alloys) in contact with oral mucosa, studies [22] showed that statistically significant differences are observed in DNA damage by commet assay [*cell olive moment:* 9.35±11.68; 68.41±26.63, respectively ($p<.05$)] as compared to what is observed for the unexposed controls; but did not show this trend for titanium tubes and brackets ($p>.05$). Moreover, when analyzing further the DNA damage by micronucleous counting after 9 months [23] of chromium/niquel-made appliances exposure in a single group homogenous sample, it has been found that non statistical differences are observed between previous and post-exposure ($p>.05$) [pre-exposure:10.6±5.7/post-exposure:9.2±6.3] (Table 2) using internal controls as the reference value. Oppositely, the cytotoxic effects represented by cellular viability in the oral mucosa cells described in T0, T1 and T2 [20] showed worsening from the reference point to the 3 ($p<.05$) and 6 months ($p>.05$) therapy compared to internal and external control groups (Table 2). Pooling the data to evaluate the overall effect of

studies at short-term was not possible because of incomparable 'controls' and statistical (chi-square < .05; I^2 > 50%) heterogeneity.

(2) Long term evaluations (>9 months) and (3) post-exposure assessment (>24 months)

Results describing DNA and cellular damage after long exposure of metallic appliances offers some kind of similar results between studies. While some researchers have found highly significant differences regarding micronucleus frequency [5 fold change; p<.0001] [21] and comets (percentage of DNA in the tail and tail length (µm)) [p=.0047] [8] in long term exposed subjects (>18 months) compared to controls. Nevertheless DNA damage to oral mucosa shows a negative regression (P>.05) compared to controls after 30 days of removing the exposure to metallic contact indicating somehow a maintained reparative nature in oral mucosa cells after induced damage by exposition to fixed metallic appliances [21] (Table 2) Similarly other authors [23] have concluded that significant differences are found (p<.05) after orthodontic therapy with fixed appliances (>15 months) reinforced with bucal miniscrews as additional metallic appliance in direct contact with oral mucosa, DNA damage was observed for both orthodontic and orthodontic + miniscrews by alkaline comet assay assessment (Table 2).

As concluding from the comparisons of the metanalysis in those comparable groups in the long term follow up an overall estimated effect that is described as compiled and fully detailed in *Appendix* Figure 4-6.

4. Discussion

To date little available information exists regarding the long term effects of metallic exposure over the oral mucosa cell's DNA, reparative capabilities and cell viability [25]. This cell population might represent a site-location preferred target for any premature DNA alteration induced by carcinogenic agents entering the body via absorption by direct contact, inhalation or ingestion [26]. Accumulated chronic or severe acute DNA and cellular damage over oral mucosal cells constitutes one of the main initiating factors contributing to a wide range of malignant lesions to occur in the oral cavity [27]. Eventually, oral mucosa remains to be persistently disrupted and mechanically irritated by the types of fixed appliances used in orthodontics, conventional

orthodontic fixed appliance consists of metallic bands/tubes, brackets, and archwires composed mainly of Nickel, Titanium, Chromium and Cobalt and other trace elements in different proportions [28]. In addition, chemical corrosion and physical damage of this metallic devices are common events in the oral cavity where alloys undergo different severe physical or chemical aggressions [29]. Several *in vitro* and *in vivo* studies have shown that metals released from orthodontic appliances are actually actually systemically distributed and may be detected in a different range in fluids and non-oral related tissues [30]. Moreover, these devices exert a maintained and localized aggression to the oral mucosa for a substantial period of time in some cases up to four years long. Despite no reaching toxic levels in saliva, blood samples or other peripheral target tissues [31-32] at systemic level it cannot be excluded that metallic ions realising by direct contact with oral mucosa concomitant with mechanical irritation might be provoking a critical absolute or additive DNA damage effect in cells of oral mucosa that may contribute to higher susceptibility to suffer from future mucosal lesions of a wide range of severity. [8]

Nevertheless, to date, notable controversy [8,20-23] is found in literature regarding the effect of such sustained damage to the oral mucosa cells at genotoxic and cytotoxic level. The present systematic review offers an analytical and critical sum up the *in vivo* effects of oral mucosa cells exposure reporting results in accordance with Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. From the initial electronic search just 6 studies fulfilled all the required criteria for eligibility. Between them 3 categories were built up based on the time of metallic exposure to the oral mucosa. As compiled in Table 2, to date two main methods are reported in selected studies for assessing DNA damage, MN counting and alkaline comet assay. Differences in sensitivity and precision of the method have been cited in the literature [21,33].

Nevertheless, despite of the method, DNA damage analysis performed in most of the studies included in the present systematic review (5 out of 6) (Table 2) revealed alterations in DNA stability by MN or ACA. Results from data analysis of the metanalysis revealed enough homogeneity for 3 of the included studies [8,21,24] [Chi-squared: 1.64; p= 0.4392] determining a global substantial effect over DNA damage in oral mucosa cells after a long-term exposure (>9months) to metallic fixed orthodontic

appliance. Current studies fail to follow up real reparative capabilities of those very long exposure subjects (>48 months) and just one research gives evidence that after 30 days of appliance removing after a long term exposure (>18 months) a regression to the basal levels is observed in DNA damage parameters. Machinery for DNA repair activates when DNA damage occurs ensuring integrity of the DNA [34-36]. However, this reparative machinery is not predictable over the persistence of DNA damage that conducts to genomic instability and may lead to potential DNA mutations [37,38]. To this regard metal ions, as those released by orthodontic appliances, have been described to alter at several levels the cellular pathways that maintain integrity and homeostasis at the genomic level [39]. Generally speaking metallic ion exposure might induce several pathogenic effects such as a stimulated response to inflammation, alteration of oxidation machinery, increased lipid peroxidation or fully alteration of the DNA repair pathways [40].

The fact that no evidence is found for any reversible dramatic changes in oral mucosa cells with current analytical methods did not justify that the releasing of metal ions and “transitory” DNA damage may be totally harmless. That might be a limitation of current analytical/diagnostic methods themselves as may have happened in much other pathology along the history. Even more, the mechanisms underlying this feature are largely unknown, but several possible pathways seem to be involved, such as the interaction of metals with DNA (crosslinks), the generation of oxidative DNA damage, or interference with DNA repair and replication processes [25]. Based on the observed effects reported in this systematic review, it might be prudent to diminish any insult, in time and severity, induced by the fixed orthodontic devices. Moreover the fact that corrosion of the metallic appliances is the main factor contributing to current registered cytotoxic and genotoxic effects, the improvement in manufacturing standards and searching for novel alloys that may prevent from corrosion may be highly desirable for ensuring a totally safe care-providing with this appliances. When evaluating cytotoxic effects, the two studies analyzing such type of damage conclude that cellular viability is highly affected (Table 2) during metallic exposure by orthodontic appliances. Whether reparative machinery may not reverse observed DNA changes and apoptosis is the fate of many of the cells in direct contact, is a matter of future study. Although many studies have evaluated metal ions released in different

fluids, few studies have quantitatively determined the ion concentrations found in oral mucosal cells [8,21]. Quantification of metallic ions concentration in oral mucosa cell population where found to be alterated in most of the studies included in the present review, despite no statistical significant correlation is observed in all of them between cellular ion content and genotoxic/cytotoxic effects [8,20].

The aims of this systematic review have been to gather the data available regarding the biological effects of exposure to metallic appliances in the orthodontic context. The results of it, however, must be taken cautiously. There is somehow heterogeneity of quality level among the studies make comparisons between them somehow difficult, but illustrates the general lack of high quality results on every one of the different studies compiled. Besides, in order to assess the quality level of the papers included different scales and methods have been described in the literature. Among them the Newcastle-Otawa scale (NOS) has been used, and although this is a widely used scale, its use is not yet validated. Some authors [41] have expressed their concern that NOS may provide a quality score that has unknown validity or that includes quality items that are even invalid and therefore may produce arbitrary results. On the other hand, in their 2007 review, Sanderson et al.[42], after identifying and analyzing the existing tools in literature for assessing quality, concluded that there was a need to agree on critical elements for assessing susceptibility to bias in observational epidemiology and to develop appropriate evaluation tools. Until that consensus is reached the present review followed current accepted guidelines by using the Cochrane risk of bias tool and the RevMan 5.2 software for data analysis as recommended by the the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement and following the Cochrane Collaboration and the Centre for Reviews and Dissemination guidance [13,14].

To our knowledge this is the first systematic critical and analytical report compiling all the available sound scientific literature regarding the genotoxic and cytotoxic effects of the short and long term exposure to a metallic irritant appliance to the oral mucosa cells.

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Figure captions

Figure 1. Flow diagram of the screening process for study selection

Figure 2. (*Supplemental Appendix*). Summ up assessment for the overall risk of bias. Other bias: represents any other apparent bias in the trial design or conduct other than the already-assessed biases in the tool [i.e., selection, performance and detection, attrition, and reporting biases]

Figure 3. (*Supplemental Appendix*). The individual domain risk of bias for each study. Colour nomenclature: green + Low risk of bias, yellow ? Unclear risk of bias, red - High risk of bias. §:assessed using the Cochrane Tool for Assessing Risk of Bias in Randomized Clinical Studies.

Figure 4. (*Supplemental Appendix*). Meta-analysis comparisons for the long term exposure to metallic ions. Galbraith Graph and analysis.

Figure 5. (*Supplemental Appendix*). Meta-analysis comparisons for the long term exposure to metallic ions. Funnel plot assessment and cumulative effect

Figure 6. (*Supplemental Appendix*). Meta-analysis comparisons for the long term exposure to metallic ions. Publication bias

Figure 7. (*Supplemental Appendix*). Meta-analysis comparisons for the long term exposure to metallic ions. Random effects model

#1 ("orthodontic subjects"[tiab] OR "orthodontic treatment"[tiab] OR orthodontic appliances[mh] OR "orthodontic therapy"[tiab] OR "orthodontic appliances"[tiab] OR orthodontic device*[tiab] OR orthodontic patient*[tiab] OR fixed appliance*[tiab])

2# (corrosion[mh] OR metals[mh:noexp] OR ions[mh:noexp] OR metal release*[tiab] OR metal content*[tiab] OR metal level*[tiab] OR metal concentration*[tiab] OR metal ion*[tiab] OR trace metal* OR ion release*[tiab] OR ion content*[tiab] OR corrosion[tiab])

3# (genotox*[tiab] OR mutag*[tiab] OR toxicity OR DNA damage OR cytotoxicity OR damage OR tissue OR fragmentation OR reactivity OR biocompatibility)

4# (oral mucosa OR mucosa OR lips OR jugal mucosa OR gingiv*)

5# (in vitro OR animal[filter])

(#1 AND #2 AND #3 AND #4 NOT #5)

("orthodontic subjects"[tiab] OR "orthodontic treatment"[tiab] OR orthodontic appliances[mh] OR "orthodontic therapy"[tiab] OR "orthodontic appliances"[tiab] OR orthodontic device*[tiab] OR orthodontic patient*[tiab] OR fixed appliance*[tiab]) AND (corrosion[mh] OR metals[mh:noexp] OR ions[mh:noexp] OR metal release*[tiab] OR metal content*[tiab] OR metal level*[tiab] OR metal concentration*[tiab] OR metal ion*[tiab] OR trace metal* OR ion release*[tiab] OR ion content*[tiab] OR corrosion[tiab]) AND (genotox*[tiab] OR mutag*[tiab] OR toxicity OR DNA damage OR cytotoxicity OR damage OR tissue OR fragmentation OR reactivity OR biocompatibility) AND (oral mucosa OR mucosa OR lips OR jugal mucosa OR gingiv*) NOT (in vitro OR animal[filter])

Table 2. General characteristics of the included studies

Study (year)	Population origin	Sample Size (F/M) [mean age]					Sample size calculation	Statistical power >80%	Dropouts reported (n)	Ortho Ap (Material)	Sample collection			DNA & Cellular damage analysis					Intracellular´s ions concentration			
											T	Type of sample	Collection Procedure	Genotoxicity			Citotoxicity	Comp analysis	Ions analysed	Detection method	Cellular mean concentration of metallic ions (Ng/mL	
		+C	C int	C ext	Ex	Ex Groups								C Int	C Ext	Ex					C int	C ext	Ex
														ACA (Olive moment)									
Martín-Camean *et al.* (2015)	Spain	§10 (5/5) [16-60]	§20 (10/10) [16-60]	20 (12/8) [16-60]	40 (19/21) [16-60]	2	+	+	-	B&T&A&Mi(SS,Ni-Ti)	T0 (15 m)	OMC (bucal mucosa)	Cytobrush	-	12,3+11,7*	Ortho: 23,4+15,08 * Ortho+MI: 25,618+17,821*	NT	NT	-	-	-	-	-
														MN (Ayyad et al.)									
Heravi *et al.*, (2013)	Iran	-	§25 (15/10) [16.3]	-	25 (15/10) [16.3]	1	NO	NO	NO	B&T&A (SS,Ni-Ti)	T0 (0 m) T1(9m)	OMC (bucal mucosa)	Scrapp metallic spatula	T0: 10.6 ± 5.7	-	T1: 9.2 ± 6.37	NT	NT	-	-	-	-	-
														MN (Fenech et al.)									
Natarajan *et al.*, (2011)	India	-	-	20 (NT) [NT]	20 (NT) [NT]	1	NO	NO	NO	B&T&A (SS,Ni-Ti)	T0 (24m + 0 m P.E) T1 (24m + 1m P.E)	OMC (lips and bucal mucosa)	DNA analysis: Scrapp metallic spatula. Cellular analysis: cytologic brush	-	T0: 53+51* T1: 32+35	T0: 259±233* T1: 48±49	NT	NT	Ni, Cr	ICP-MS		Ni T0: 3.86+2.17 Ni T1: 3.48+1.55 Cr T0: 2.71+1.73 Cr T1:2.26+1.73	Ni T0:4.09+3.2 Ni T1:3.83+1.9 Cr T0: 3.63+3. Cr T1:2.94+1.9
														ACA (Tice et al) (Olive moment)									
Hafez et al., (2011)	Egypt	-	§28(6/22) [20.2]	18 (8/10) [21.6]	28(6/22) [20.2]	4	NO	NO	Yes (8)	B&T&A (SS,Ni-Ti, Ti)	T0 (0 m) T1(3m) T2(6 m)	OMC (bucal mucosa)	Scrapp wooden tonge depressor (Nia et al)	T0: 108+30.9 T1: 50.9+27.1* T2: 101.1+33.6		T0: 125.6+46.05 T1: 108.4+54.06* T2: 98.8 +33.7	TBDT	NT	Ni, Cr	GF-AA	NiT0: 0.31+60.17 NiT1:0.29+60.16 NiT2 0.29+60.12	-	Ni T0: 0.51 +6 NiT1: 0.67 +6 NiT2: 0.78 +6 CrT0 0.30 + 6 CrT1 0.40 +6 Cr T2 0.58+6
														ACA (Olive moment)									
Fernandez-Miñano *et al.*, (2011)	Spain	+	§15 (NT) [12-16]	-	15 (NT) [12-16]	3	NO	NO	NO	B&T (SS,Ni-Free, Ti)	T0 (0 m) T1(1m)	OMC (cheek mucosa)	Interdental brush	-	-	SS: 69.35+11.68* Ni-Free: 68.41±26.63*	NT	NT	Ni, Co, Cr, Ti, Fe, Mn, Mo	IPC-MS	Ti47 0.98 ± 0.64 3.04 ± 1.67 1.00 ± 0.26 0.82 ± 0.30 Cr 0.00 ± 0.00 0.0 0.00 Fe 1.95 ± 1.29 2.01 ± 0.46 1.24 ± 0.79 5.36 ± 2.44		Ni.44 ± 2.79 0.04 ± 0.07 0.00 ± 0.00 0.0 Mo 0.13 ± 0.1 Fe 1.95 ± 1.29 2.01 ± 0.46 1.24 ± 0.79 5.36 ± 2.44
														ACA (Speit et al) (Olive moment)									
Faccioni *et al.*, (2003)	Italy	-	-	30(13/17) [12-33]	55(33/32) [12-35]	1	NO	+	NO	B&T&A (SS,Ni-Ti, Ti)	T0 (0 m) T1(24-48 m)	OMC (cheek mucosa)	Interdental brush	11.43±6.58*		17.62+10.08*	TBDT	NT	Ni, Co	ICP-MS	Ni: 0.72+ 0.62* Co: 0.20+0.091*		Ni: 2.52+1.76* 0.56+0.40*

§: same sample as experimental group;+C: positive control for DNA damage; Ortho Ap: Type of orthodontic fixed appliance; m: month; OMC: Oral mucosa cellsFe, Iron; Ni, nickel; Cr, chromium; Mn, manganese; Mo, molybdenum; Ti, titanium; Co, cobalt; copper; Si, silicon; Al, aluminum; P, phosphorous; S, sulfur; C, carbon; O, oxygen; H, hydrogen; N, nitrogen.B&T&A&Mi: Brackets,tubes, archwires and miniscrew; SS: Stainess Steel; Ni-Ti: Niquel Titanium; F: female; M: male; T: Lenght of orthodontic treatm when collected; Cint: internal control group; Cext: external control group; Ex: Experimental group; Comp analysis: Complementary analysis; MN: Micronucleous; ACA: Alkaline comet assay; TBDT: Trypan Blue dye test; NT: non tested; GF-AA: Graphite furna atomic absorption; P.E: post-exposure; *:p<.05

Ayyad SBA, Israel E, Setouhy ME, Radwan G, Mohamed MK, Loffredo CA. Evaluation of Papanicolaou stain for studying micronuclei in buccal cells under field conditions. Acta Cytol 2006;50: 398-402.

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Nia et al: Nia A, Van Straaten H, Godschalk R, Van Zandwijk N, Balm A, Kleinjans J, et al. Immunoperoxidase detection of polycyclic aromatic hydrocarbon-DNA adducts in the mouth floor and buccal mucosa cells of smokers and nonsmokers. Environ Molec Mutagen 2000;36:127-33.

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Speit G, Hartmann A. The comet assay (single-cell gel test): a sensitive genotoxicity test for the detection of DNA damage and

Flow diagram screening process

IDENTIFICATION

PUBMED

#1:27902
#2:103560
#3:3853648
#4: 321858
#1 AND #2 AND #3 AND #4 NOT#5:17

129 EMBASE
#*#1

1255 SCOPUS
#*1

3741 SCIRUS
##1

4 Cochrane Library
#**1: 21
#**2: 4

2 Hand Search

SCREENING

1280 duplicated references

580 Titles and abstract read

ELIGIBILITY

550 Excluded on basis of abstract

-Inclusion criteria for article type not met (such as editorial, opinion, review, protocol, response, meeting abstract)
-Inclusion criteria for study design not met
-Not outcome of interest

10 Full article read

0 On basis of reference lists of retrieved articles

INCLUDED

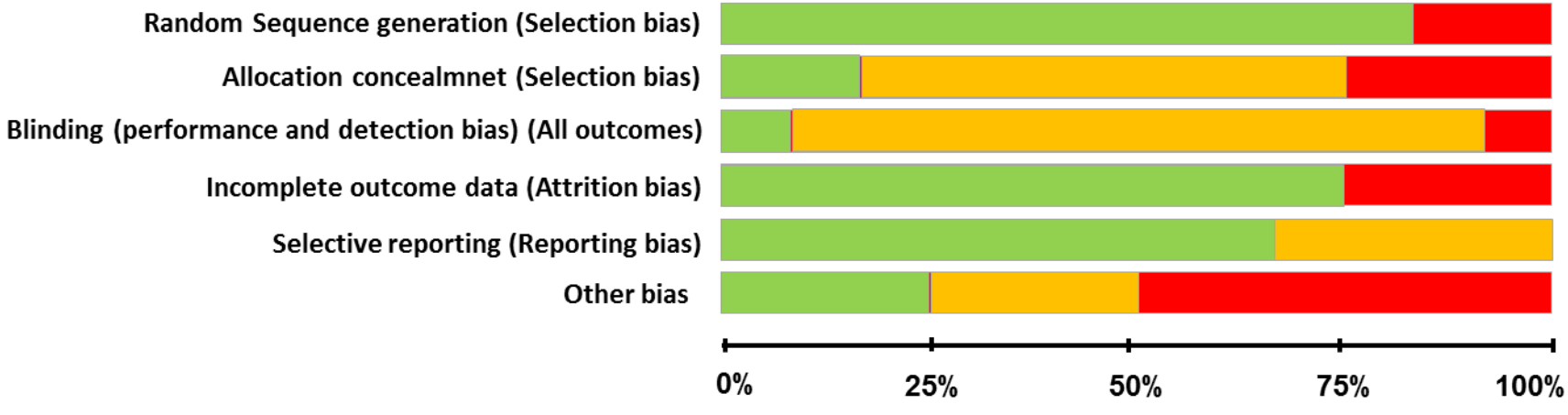
4 Excluded on basis of article

-Inclusion criteria for study design not met
-Not outcome of interest

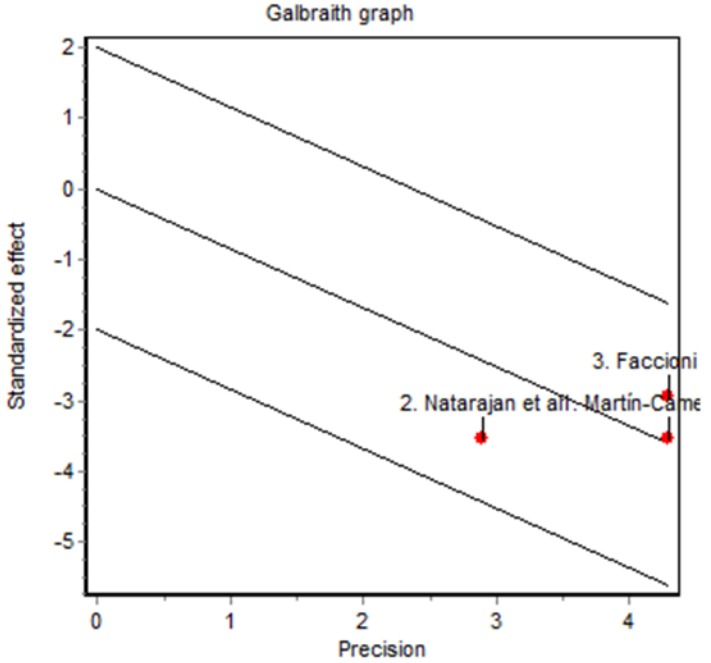
6 Included

6 Final Included

0 Included

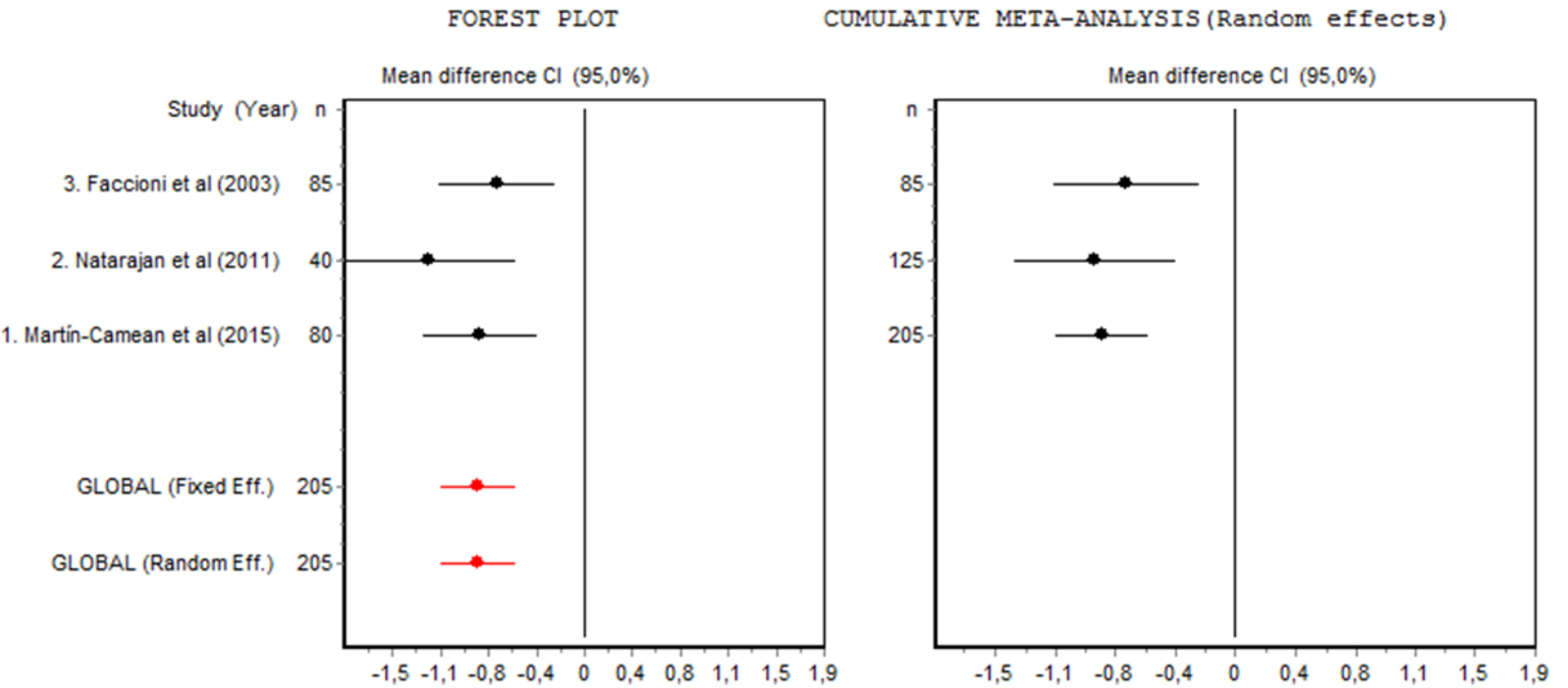

Random Sequence generation (Selection bias)
Allocation concealmnet (Selection bias)
Blinding (performance and detection bias) (All outcomes)
Incomplete outcome data (Attrition bias)
Selective reporting (Reporting bias)
Other bias
0%
25%
50%
75%
100%


Random Sequence generation (selection bias)
Allocation concealmnet (selection bias)
Blinding (performance bias and detection bias) (All outcomes)
Incomplete outcome data (attrition bias)
Selective reporting (reporting bias)
Other bias
§ Martin-Camean et al, 2015
LOW
§ Heravi et al., 2013
LOW
§ Natarajan et al., 2011
HIGH
§ Hafez et al., 2011
HIGH
§ Fernandez-Miñano et al., 2011
HIGH
§ Faccioni et al., 2003
HIGH



INDIVIDUAL AND COMBINED RESULTS

Study	Year	n	d	CI(95,0%)		Weights(%) Fixed eff.	Random eff.
3. Faccioni et al	2003	85	-0,6868	-1,1435	-0,2301	40,6658	40,6658
2. Natarajan et al	2011	40	-1,2184	-1,8932	-0,5435	18,6219	18,6219
1. Martín-Camean et al	2015	80	-0,8225	-1,2789	-0,3660	40,7123	40,7123
Fixed effects		205	-0,8410	-1,1322	-0,5498		
Random effects		205	-0,8410	-1,1322	-0,5498		


FOREST PLOT
CUMULATIVE META-ANALYSIS(Random effects)
Mean difference CI (95,0%)
Mean difference CI (95,0%)
Study (Year)
n
3. Faccioni et al (2003)
85
2. Natarajan et al (2011)
40
1. Martín-Camean et al (2015)
80
GLOBAL (Fixed Eff.)
205
GLOBAL (Random Eff.)
205
n
85
125
205
-1,5 -1,1 -0,8 -0,4 0 0,4 0,8 1,1 1,5 1,9
-1,5 -1,1 -0,8 -0,4 0 0,4 0,8 1,1 1,5 1,9

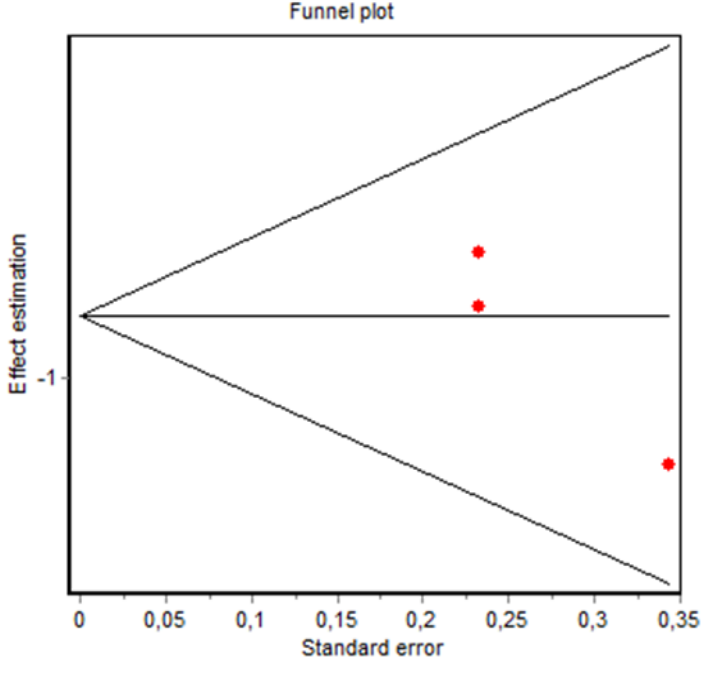
PUBLICATION BIAS

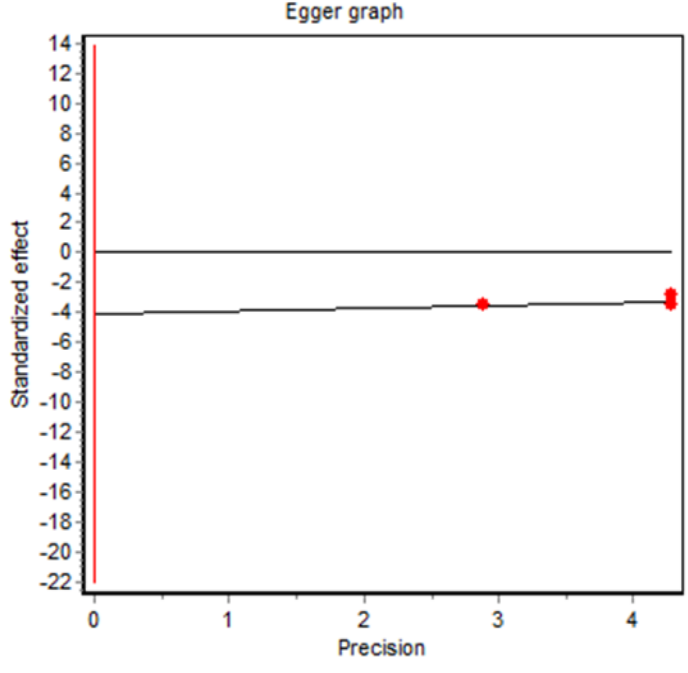
Begg test

Z statistic	p-value
0,0000	1,0000

Egger test

t statistic	df	p-value
-2,9368	1	0,2089

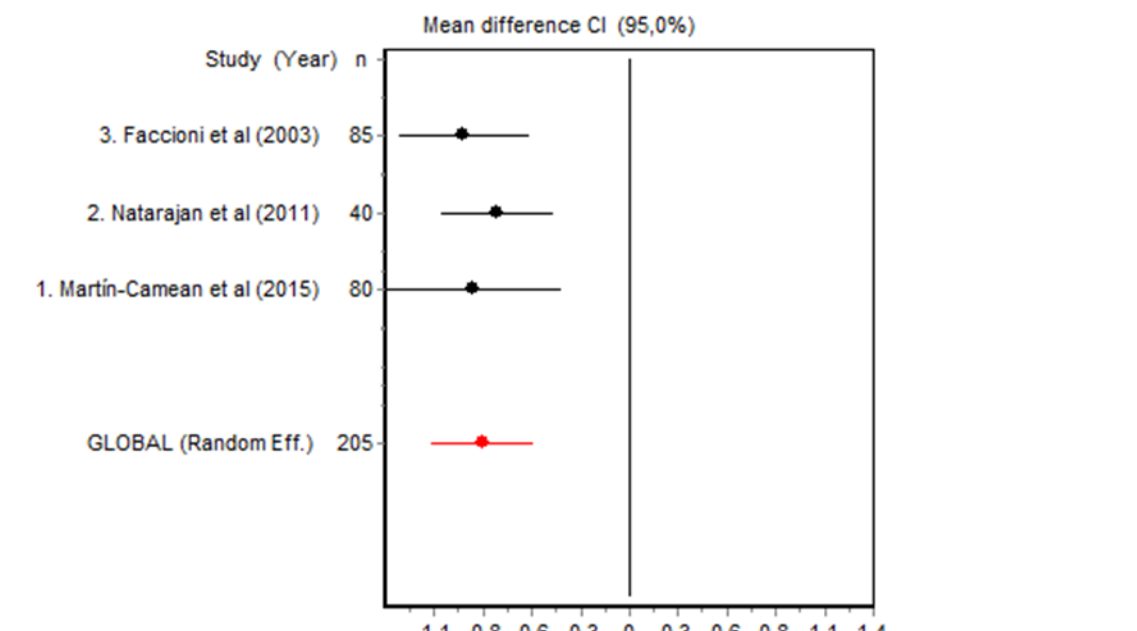




RANDOM EFFECTS MODEL

Omitted study	Year	n	d	CI(95,0%) Lower limit	CI(95,0%) Upper limit	Relative change (%)
3. Faccioni et al	2003	120	-0,9467	-1,3248	-0,5686	12,57
2. Natarajan et al	2011	165	-0,7547	-1,0775	-0,4318	-10,27
1. Martín-Camean et al	2015	125	-0,8921	-1,3995	-0,3848	6,08
GLOBAL		205	-0,8410	-1,1322	-0,5498	

Influence graph



V. DISCUSIÓN GENERAL / GENERAL DISCUSSION

Tal y como se ha expuesto previamente en la Introducción, los estudios sobre biocompatibilidad de los materiales ortodóncicos, que incluyan tanto estudios de liberación de iones metálicos constituyentes de los mismos a lo largo del tratamiento, como evaluación de los efectos potenciales *in vivo*, son de interés creciente.

En las revisiones llevadas a cabo, se ha realizado una puesta al día de la literatura científica sobre la liberación *in vitro* e *in vivo* de iones metálicos componentes de la aparatología ortodóncica y sus efectos tóxicos, con especial interés en los estudios de citotoxicidad y genotoxicidad llevados a cabo hasta la fecha actual. Los estudios *in vitro* indican que dada la diversidad en la composición de los materiales y las técnicas de fabricación de los mismos, es necesario una evaluación de su seguridad caso por caso. Entre las lagunas detectadas destaca que el número de estudios de toxicidad *in vivo*, tanto de liberación de iones como de citotoxicidad y genotoxicidad, es escaso (en comparación con *in vitro*) siendo prioritario el emprenderlos, ya que proporcionan una información más adecuada de los efectos de los materiales de ortodoncia en un escenario real. En los estudios *in vivo* de liberación de iones metálicos no se ha constatado la aplicación de métodos validados de determinación, y dados los resultados contradictorios existentes, deben llevarse a cabo estudios de monitorización (especialmente de Ni y Cr) que permitan investigar las relaciones causa-efecto de interés, así como investigaciones encaminadas a conocer los mecanismos involucrados en los efectos tóxicos observados (por ej., efectos sobre el ADN por mecanismos de estrés oxidativo). Además, se ha observado que la mayoría de los estudios experimentales se han llevado a cabo con aparatología ortodóncica tradicional, pero sería prioritario el avance del conocimiento en nuevos materiales, o nuevos procedimientos, o investigar nuevas matrices, sobre todo las que permitan una evaluación a largo plazo de la potencial acumulación de

elementos metálicos en pacientes en tratamiento de ortodoncia.

La revisión sistemática llevada a cabo sobre células de la mucosa oral, siguiendo las guías PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) hasta fecha actual (29 abril 2015) permitió recopilar toda los efectos cito/genotóxicos, se seleccionaron 17 artículos y 6 estudios cumplieron los criterios adecuados. Además de las diferencias significativas observadas en el muestreo y métodos empleados en los diversos estudios, algunos de ellos (2-3) evaluaron efectos después de eliminar las aplicaciones ortodóncicas, concluyendo que los efectos sobre el ADN o a nivel celular no se diferenciaron de forma significativa con respecto a los controles. Concluimos por tanto, la necesidad de llevar a cabo de forma adicional rigurosos estudios clínicos aleatorios para explorar la continuidad del daño inducido por las aplicaciones ortodóncicas en la mucosa oral, sobre todo en el tramo de población joven (12-26 años).

Es por todo ello, por lo que decidimos realizar una serie de estudios encaminados, por una parte, al desarrollo y validación de métodos analíticos para cuantificar la liberación de iones metálicos en células de la mucosa oral en pacientes bajo tratamiento de ortodoncia, y por otro lado, investigar la validez del empleo de nuevas matrices como el pelo, para evaluar la acumulación de dicha liberación, tras exposición a largo plazo.

1. DESARROLLO Y VALIDACIÓN DE MÉTODOS DE DETERMINACIÓN DE IONES METÁLICOS EN CÉLULAS DE LA MUCOSA ORAL DE PACIENTES EN TRATAMIENTO ORTODÓNCICO.

La liberación de metales en células de la mucosa oral, con prolongado contacto con las aplicaciones ortodóncicas fijas, ha sido escasamente investigada (Faccioni y col., 2003; Amini y col., 2008; Hafez y col., 2001; Natarajan y col., 2011; Fernández-Miñano y col., 2011), y a pesar de las ventajas de esta matriz en

comparación con la saliva, no existen métodos validados de determinación de metales en dichas células. Además, no se han llevado a cabo estudios de valoración de elementos como Zirconio (Zr) y Vanadio (V), constituyentes de materiales de uso en ortodoncia (arcos), algunos muy recientes como "Gum metal" (a base de Ti_3(Ta+Nb+V) +(Zr,Hf)+O (Bishino, 2003).

De forma general, los enfoques clásicos para la validación de métodos analíticos sólo se basaban en la comparación entre los valores medidos y los de referencia, sin tener en cuenta la importancia de la precisión y reproducibilidad del método intra- e interlaboratorios, incluyendo estudios de robustez, lo cual es imprescindible para la "transferencia del método" (González col., 2010). Por todo ello, desarrollamos un método rápido y sensible de determinación simultánea de Ti, V y Zr en células de mucosa oral de pacientes bajo tratamiento ortodóncico, mediante ICP-MS, que fue validado mediante estándares de validación, de acuerdo con González y col., (2010). El método de preparación de las muestras incluyó la digestión de las células extraídas a partir de minicepillos en tubos de centrifuga (con modificaciones sobre métodos anteriores) con 10 mL de agua desionizada y 100 µL de HNO_3 Plasmapure 65%, calentamiento en baño de agua (80ºC) durante 60 minutos, sonicación en baño de ultrasonidos durante 5 minutos, y enfriamiento a temperatura ambiente. El método fue validado, de forma que las ecuaciones de regresión se calcularon a partir de disoluciones estándares preparadas en la misma matriz desprovistas de células de mucosa oral, de forma que los rangos de concentraciones fueron amplios, entre 0,5-50 ng/mL para Zr y de 5.0-50.0 ng/mL para Ti y V, obteniéndose coeficientes de correlación que oscilaron en un rango entre 0,9965 (Ti) y 0,9994 (Zr). Los límites de detección (LOD) (0,9, 2,8 y 0,4 ng/mL para Ti, V y Zr, respectivamente) y de cuantificación (LOQ) (1,8, 3,4 y 0,7 ng/mL en el caso de Ti, V y Zr, respectivamente) fueron asimismo muy adecuados, siendo la primera vez que se publican en la bibliografía

científica. Éstos fueron similares a los encontrados por Amini y col. (2008) para Ni y Cr en células de mucosa oral, determinados por otra técnica, GF-AAS. Las recuperaciones obtenidas a tres niveles de concentraciones ensayadas oscilaron entre 101-108% en el caso de Ti, 92-104% para Zr y entre 98-111% para el V, cumpliéndose en todos los casos los rangos aceptables publicados en función de las concentración de analito (González y col., 2010; Huber, 1998). Los valores de repetibilidad y de precisión intermedia (%RSD) fueron satisfactorios. Asimismo, la combinación de tres factores variables en el proceso de digestión (tiempo empleado de calentamiento; volumen de agua desionizada para diluir las muestras; y volumen de ácido nítrico Plasmapure 65% empleado) demostró la robustez del método para ser reproducido bajo diferentes condiciones sin sufrir variaciones significativas en los resultados.

El método validado se aplicó con éxito a la determinación de estos tres elementos en 40 pacientes, 20 de los cuales estaban bajo tratamiento de ortodoncia durante un promedio de 13-15 meses, y 20 individuos controles. En el caso del Ti, no hubo diferencias significativas en los contenidos obtenidos en los grupos experimental y control, y solo se detectaron cantidades traza de Zr en el grupo de ortodoncia, no existiendo tampoco diferencias con el grupo control. Los resultados traza del Zr fueron consistentes con la propia composición de los materiales empleados durante el tratamiento, que fue determinada mediante Micro-fluorescencia de Rayos X (μFRX). La no existencia de diferencias significativas de liberación de metales entre grupo control y pacientes en tratamiento de nuestro estudio, concuerda con los resultados previos obtenidos para Ni y Cr por Natarajan y col. (2011). Sin embargo, otros autores, sí demuestran diferencias significativas, con una mayor liberación de Ni y Co en pacientes ortodóncicos (Faccioni y col., 2003), de Ti y Mn (Fernández-Miñano y col., 2011), o de Ni y Cr tras 6 meses de tratamiento (Hafez y col., 2011). La no

detección de V en ambos grupos se justifica igualmente por la composición de la aparotología ortodóncica empleada (solo existente en un porcentaje pequeño 0,18-1.19% en bandas), pero es muy adecuado el método para monitorizar materiales de empleo emergente en ortodoncia.

Debido a que los elementos que más abundan en brackets, bandas y arcos son Ni (15-54% en arcos), Co (40-60%), Cr (20-30%), en diferentes porcentajes, así como Fe y Cu, decidimos desarrollar y validar un procedimiento de determinación de estos elementos en células de la mucosa oral de pacientes en tratamiento ortodóncico mediante ICP-MS, basándonos en el método de tratamiento de muestras anterior. Comprobamos en ensayos preliminares la lixiviación de Co, Cr, Cu y Ni a partir de minicepillos y de cepillos interdentales con partes metálicas, por lo que empleamos en este trabajo cepillos interdentales de goma para la toma de muestra, sin contenidos metálicos. De nuevo, el método propuesto fue validado, de forma que las ecuaciones de regresión a partir de estándares preparados en la misma matriz fueron lineales entre 2,0-100,0 ng/mL para todos los elementos. Los LOD obtenidos de 0,10, 0,38, 0,49 y 0,67 ng/mL, para Co, Cr, Cu y Ni, respectivamente, fueron muy satisfactorios, así como sus respectivos LOQ de 0,20, 1,13, 0,98 y 1,81 ng/mL. Las recuperaciones también fueron excelentes, variando en el caso del Co entre 104-109%, entre 103-107% para Cr, 106-113% para Cu y 84-110% para el Ni. La precisión intermedia (RSD%), a tres niveles de concentración, fue también muy adecuada, y el método demostró también su robustez para la determinación de los cuatro elementos considerados. La aplicación del método validado se llevó a cabo de forma similar al estudio anterior, de 40 pacientes, 20 controles y 20 pacientes en tratamiento ortodóncico, cuya aparatología ortodóncica se analizó por μFRX. Su aplicación nos permitió tener más datos *in vivo* sobre la liberación de estos metales potencialmente liberados, de forma que en este trabajo se encontraron diferencias

estadísticamente significativas en los contenidos de todos los elementos, en comparación con el grupo control (entre 2-veces y 13,5 veces para Cu y Co, respectivamente). Ello concuerda con los resultados de Faccioni y col. (2003) que encontraron contenidos 3,4 veces más elevados de Ni y 2,8 veces superiores de Co en pacientes tras 2-4 meses de tratamiento ortodóncico, y con los resultados de Hafez y col. (2011) que informan de mayores concentraciones de Ni y Cr en pacientes tras 6 meses de tratamiento. De nuevo, los escasos resultados encontrados en la bibliografía resultan ser contradictorios, pues Natarajan y col. (2011) no encontraron diferencias en las concentraciones de Ni y Cr. Asimismo, Amini y col., (2008) informan de mayores concentraciones de Ni en pacientes tratados, pero no de Co y de Cr, y Fernández-Miñano y col. (2011) detectaron Cr y Fe. Indicar que en nuestro estudio ninguno de los pacientes manifestó síntomas durante los 1,5-2 años de tratamiento, y que al igual que en los estudios *in vivo* publicados hasta la fecha, las concentraciones detectadas son muy inferiores a las ingestas diarias tolerables (IDT o TDI) publicadas para Ni, Co, o los niveles máximos de ingesta tolerables establecidos para Cu y Cr (III) (EFSA, 2015; EGVM, 2003; SCF, 2003 a,b).

2. LA LIBERACIÓN IÓNICA EN PELO DEL CUERO CABELLUDO DE PACIENTES EN TRATAMIENTO DE ORTODONCIA: IDONEIDAD DE DICHA MATRIZ Y BIOMONITORIZACIÓN DE IONES METÁLICOS

Ya que los metales no son biodegradables y se acumulan en tejidos, se ha demostrado que el pelo humano es un vehículo adecuado de acumulación y excreción de metales pesados (hasta concentraciones 10 veces superiores en comparación con sangre y orina), debido a la presencia de cisteína (14%), por la producción de enlaces entre los cationes metálicos y los grupos sulfuro de la matriz de queratina del pelo (Olmedo y col., 2010). De hecho, se considera esta

matriz no invasiva uno de los materiales biológicos más importantes de monitorización ambiental (Morton y col., 2002). Dicha matriz presenta numerosas ventajas para su uso en biomonitorización humana, tales como: fácil obtención de la muestra, bajo coste, fácil transporte y almacenamiento, no sufre cambios durante el periodo transcurrido entre muestreo y análisis, y además, proporciona información sobre exposición a corto y a largo plazo (Barbosa y col., 2005; Angerer y col., 2007; Zhang y col. 2007; Gil y col., 2011). A pesar de ello, los procedimientos analíticos para la determinación de metales en dicha matriz son escasos, existiendo únicamente un estudio preliminar (procedimiento no validado, muy escaso número de pacientes) que demuestra que no existían diferencias significativas en el contenido de algunos elementos metálicos en pelo de pacientes bajo tratamiento de ortodoncia.

En este trabajo, nos propusimos, mediante la aplicación de procedimientos analíticos validados previamente (Olmedo y col., 2010), determinar mediante Espectrometría de absorción atómica (AAS) los contenidos de Cu, Fe (variedad llama), y Cr, Mn y Ni (cámara de grafito con corrección de fondo Zeeman, GFAAS) en pelo de cuero cabelludo de 70 pacientes en tratamiento de ortodoncia, en comparación con una población constituida por 56 controles. Sólo se demostraron incrementos significativos de Mn en los pacientes con respecto a los controles, y siendo los contenidos de la misma magnitud que los informados en otras poblaciones controles, concluimos que no se han encontrado riesgos por una acumulación de iones metálicos por el tratamiento. Se estudió la influencia de factores individuales (sexo, edad) y se demostró una mayor acumulación en pelo de mujeres, lo que concuerda con otros estudios anteriores en poblaciones sin tratamiento ortodóncico (Senofonte y col., 2000; Gil y col., 2011). Además, en general no había variaciones en la acumulación metálica con la edad, a excepción de las concentraciones superiores de Mn

encontradas en jóvenes pacientes (< de 20 años). La influencia de la edad ha sido estudiada en poblaciones control por diversos autores y los resultados han sido contradictorios, a veces los contenidos aumentan con la edad (Cu en mujeres, Co, Zn), mientras que en otros elementos metálicos el perfil de acumulación no se ve influido por la edad (Senofonte y col., 2000; Chojnacka y col., 2006). Se detectaron correlaciones positivas entre Cu/Mn, Cu/Ni y Fe/Ni, que pueden ser explicadas por sinergismos existentes entre ellos (Chojnacka y col., 2006), probablemente por su similitud química; otras interacciones se pueden producir entre Fe y Cr, de acuerdo con Mikuliewicz y col., (2011). Por tanto, este estudio nos ha permitido generar valores de metales en esta matriz que pueden ser muy útiles con fines comparativos, y se concluye que la determinación *in vivo* de iones metálicos propuesta en cabellos proporciona un buen método de biomonitorización de la liberación de estos elementos.

3. LIBERACIÓN DE METALES *IN VIVO* Y POTENCIAL GENOTÓXICO DERIVADO DEL EMPLEO DE MICROTORNILLOS, EN CELULAS DE LA MUCOSA ORAL.

Una vez demostrada la liberación de ciertos iones metálicos en pacientes sometidos a aparatología ortodóncica convencional, nos interesó investigar si la aplicación de microtornillos, ampliamente utilizados por su efectividad como dispositivos de anclaje, pudiera aportar alguna liberación adicional de metales *in vivo* en la matriz estudiada, células de la mucosa oral, dada la inexistencia de estudios al respecto en humanos. Investigamos la liberación de Al, Cu, Cr, Mn, Ni, Ti y V en dicha matriz, en 20 pacientes ortodóncicos (brackets, arcos y bandas), en comparación con 20 pacientes tratados adicionalmente con microtornillos, además de 20 individuos control, mediante ICP-MS. La elección de los elementos se hizo en base a la composición de la aparatología empleada,

determinándose la composición de los mismos mediante μFRX, destacando los porcentajes de Ti (88%), seguido de Al (7%) y V (4%).

Realizamos un estudio de liberación de metales a partir de los microtornillos *in vitro*, siguiendo las normas ISO 10993-12, durante 24 y 72 horas en saliva artificial. Se cuantificaron mediante ICP-MS, y se comprobó que se podían liberar cantidades significativas de Al y Ni después de 24h y 72 horas, por lo que pueden incluir en la potencial liberación *in vivo* de estos metales. Para la evolución de la liberación de iones metálicos *in vivo* se modificó el procedimiento de preparación de las células orales de los dos métodos anteriormente validados, y se redujo el ruido de fondo de los reactivos e instrumental (mediante triple destilación del ácido nítrico empleado, agua tipo I a partir de agua Milli-Q, utilización de material plástico de laboratorio en lugar de vidrio, y autoaspiración del nebulizador), lo que supuso una mejora en los LODs y LOQs de los elementos implicados.

La liberación de iones metálicos siguió el siguiente orden ascendente: Cr < Ni < Ti < Cu < Al, y no se detectaron V y Co. La liberación estadísticamente significativa de ión Ni en los dos grupos de pacientes tratados (ortodóncicos y ortodóncicos+microtornillo) en comparación con el grupo control puede explicarse porque este metal es un constituyente común en la aparatología ortodóncica, y como se ha comentado, diversos autores también demuestran dicha liberación significativa en células de la mucosa oral (Amini y col., 2008; Hafez y col., 2011), aunque Natarajan y col (2011) no encontraron diferencias después de 30 días del cementado, por lo que el periodo de estudio considerado resulta ser un factor primordial. En relación a los contenidos de Cu, hubo incrementos del mismo en el grupo de ortodoncia pero no en el de ortodoncia+microtornillo, existiendo una gran variabilidad en los resultados obtenidos, y una escasez de datos en la bibliografía consultada. Con respecto al Cr, no se demostró liberación

significativa en el tratamiento ortodóncico, ni tampoco con el empleo de microtornillos. Los resultados recogidos en la bibliografía respecto a la liberación de Cr son de nuevo contradictorios (Amini y col. 2008; Natarajan 2011, Hafez y col. 2011; Fernández-Miñano y col., 2011). En este trabajo se informa por primera vez de la liberación de Al (presente en el microtornillo), no existiendo diferencias significativas en los tres grupos estudiados. Se demuestra una interrelación positiva en la liberación de Al/Ti, por primera vez, que puede explicarse en parte por el origen común de ambos elementos, el microtornillo, o efectos sinérgicos entre ambos. No se demostraron correlaciones estadísticas entre liberación de metales y diversos factores clínicos o de tratamiento, como: sexo, presencia del microtornillo, tipo de arcos y su colocación etc. quizá debido en parte a las limitaciones del tamaño muestral. En definitiva, la incorporación de microtornillos no implica según nuestro estudio un incremento significativo de liberación metálica, y se recomiendan nuevos estudios, con un mayor número de pacientes y monitorización paulatina de dicha liberación.

Una vez demostrada la liberación iónica tanto a partir de aparatología ortodóncica como con el uso de microtornillos, nos preguntamos por los efectos potenciales que pudieran derivarse de dicha liberación (a nivel celular, ya que no se observaron efectos en los pacientes tratados), como efectos genotóxicos, habida cuenta de que varios elementos metálicos han demostrado genotoxicidad, como Ni, Cr y Co. Además los limitados datos existentes *in vivo* se refieren a brackets, arcos y bandas (Faccioni y col., 2003; Westphalen y col., 2008; Angelieri y col., 2011b; Fernández-Miñano y col., 2011; Hafez y col., 2011; Natarajan y col., 2011; Ortiz y col., 2011), no existiendo datos acerca de los microtornillos. Tras modificar y optimizar las condiciones de aplicación del ensayo cometa alcalino en células de la mucosa oral debidas a otros autores (Collins y col., 1997; Szeto y col., 2005; Hafez y col., 2011; Collins y Azqueta, 2012), se aplicó nuestro método

de forma satisfactoria a los tres grupos de pacientes anteriormente mencionados, incluyendo un grupo control positivo, constituido por fumadores. Los valores del porcentaje de ADN en la cola analizados en los grupos tratados (ortodoncia y ortodoncia+microtornillo) fueron significativamente superiores (2 veces) a los del grupo control, aunque los microtornillos no aportaron un daño genotóxico adicional. Se comprobó, dentro de las limitaciones del tamaño muestral, la no influencia del sexo sobre la genotoxicidad de las aplicaciones ortodóncicas, aunque Slyskova y col. (2014) han indicado que el sexo puede estar asociado con un nivel de daño en el ADN superior en mujeres, en general. Nuevos estudios *in vivo* son necesarios en esta dirección para confirmar estos resultados preliminares, que incluyan un mayor número de pacientes, con microtornillos de distinta composición, y monitorización del potencial daño.

VI. CONCLUSIONES / CONCLUSIONS

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se ha llegado a las siguientes conclusiones:

PRIMERA. La revisión de actualización de la bibliografía científica ha puesto de manifiesto la necesidad de llevar a cabo estudios de liberación *in vitro* caso por caso, según protocolos establecidos. El número de estudios *in vivo*, tanto de liberación de iones como de citotoxicidad y genotoxicidad, es escaso (en comparación con *in vitro*) siendo prioritario el emprenderlos, siguiendo métodos validados de determinación, que incluyan estudios de monitorización durante el tratamiento de ortodoncia, así como investigaciones encaminadas a conocer los mecanismos involucrados en los efectos tóxicos observados. Es prioritario aplicar dichos estudios en materiales y procedimientos emergentes, y utilizar matrices que permitan una evaluación a largo plazo de la acumulación de metales en pacientes en tratamiento de ortodoncia (ej., pelo). De la revisión sistemática se concluye asimismo la necesidad de llevar a cabo estudios clínicos aleatorios para determinar la continuidad de los daños genotóxicos inducidos durante el tratamiento ortodóncico en población joven.

SEGUNDA. Hemos desarrollado y validado un método analítico, sensible, reproducible, preciso y robusto, que constituye una herramienta muy útil para la determinación de forma simultánea de la liberación de Co, Cr, Cu, Ni, Ti, V y Zr a partir de aparatología ortodóncica *in vivo*, en células de mucosa oral de pacientes tratados, por espectrometría de masas con plasma acoplado inductivamente (ICP-MS). El método fue lineal en un rango de concentraciones entre 0,5-100 ng/mL. Se obtuvieron LODs y LOQs aceptables, con recuperaciones en un rango de 84- 111%, y los valores de repetibilidad y precisión intermedia (%RSD) fueron satisfactorios.

TERCERA. La aplicación del método validado en células de la mucosa oral de 40 pacientes, 20 en tratamiento de ortodoncia y 20 individuos controles, ha demostrado incrementos significativos de las concentraciones de Co, Cr, Cu y Ni en los pacientes con aparatología ortodóncica, aunque las concentraciones halladas son muy inferiores a las Ingestas diarias tolerables de Ni y Co, o los niveles máximos de ingesta tolerables establecidos para Cu y Cr (III).

CUARTA. La determinación mediante métodos validados por Espectrometría de absorción atómica (AAS) de los contenidos de Cu, Cr, Fe, Mn y Ni en pelo de cuero cabelludo de 70 pacientes en tratamiento de ortodoncia, en comparación con una población constituida por 56 controles, demostró sólo incrementos significativos de Mn en los pacientes con respecto a los controles. Dado que todas las concentraciones halladas fueron de la misma magnitud que las ya informadas en otras poblaciones controles, concluimos que no se han encontrado riesgos por una acumulación de iones metálicos por el tratamiento de ortodoncia en la población estudiada.

QUINTA. En el estudio de biomonitorización en pelo, se ha comprobado una mayor acumulación del contenido metálico en pelo de mujeres, en comparación con hombres. En relación con la edad, sólo se detectaron concentraciones superiores de Mn en pacientes jóvenes (< de 20 años). Se detectaron correlaciones positivas entre Cu/Mn, Cu/Ni y Fe/Ni, que pueden ser explicadas por sinergismos existentes entre ellos, probablemente por su similitud química. El estudio nos ha permitido generar valores de metales en esta matriz que pueden ser muy útiles con fines comparativos, concluyéndose la validez de la determinación de iones metálicos en cabello como método de biomonitorización de la liberación de estos elementos.

SEXTA. La liberación de iones metálicos, Al, Cu, Cr, Mn, Ti y V en 20 pacientes con tratamiento de ortodoncia convencional, en comparación con 20 tratados adicionalmente con microtornillos e individuos control, ofreció el siguiente orden ascendente de concentraciones: Cr < Ni < Ti < Cu < Al, no detectándose V y Co. Se demostró un incremento significativo de la liberación de ión Ni en los dos grupos de pacientes tratados (ortodóncicos y ortodóncicos+microtornillo), y de Cu sólo en pacientes bajo tratamiento de ortodoncia, en comparación con la población control. Se demostró una correlación positiva en la liberación de Al/Ti, mientras que no se pudieron establecer correlaciones estadísticas entre liberación de metales y diversos factores clínicos o de tratamiento como: sexo, presencia del microtornillo, tipo de arcos y su colocación.

SÉPTIMA. La optimización de las condiciones del ensayo cometa alcalino en células de la mucosa oral y su aplicación en pacientes con tratamiento de ortodoncia, y ortodoncia+microtornillo para valorar daños genotóxicos potenciales, mostró incrementos significativos de los valores del porcentaje de ADN en cola en dichos grupos tratados, en comparación con un grupo control, aunque los microtornillos no aportaron daño genotóxico adicional. Se comprobó, dentro de las limitaciones del tamaño muestral, la no influencia del sexo sobre la genotoxicidad de las aplicaciones ortodóncicas.

OCTAVA. Finalmente, y en base a todo ello, se propone la biomonitorización de metales constituyentes de aparatología ortodóncica en células de la mucosa oral y en pelo de pacientes en tratamiento, como estrategia adecuada para evaluar nuevos materiales y procedimientos. Igualmente, el ensayo cometa *in vivo* ha demostrado ser un método válido para valorar la inducción de efectos genotóxicos por estos materiales.

From the results obtained in the present Doctoral Thesis, the following conclusions have been derived:

FIRST. The review to up to date the scientific literature has shown that it is necessary to perform, case by case, *in vitro* studies on the metal release from orthodontic appliances following standardized procedures.The number of *in vivo* studies, dealing with ions release as well as cytotoxicity and genotoxicity, is scarce (in comparison to *in vitro* studies). Therefore, they should be performed following validated analytical methods, including monitoring along the treatment period. Further research in order to elucidate the toxic mechanisms involved in the toxic effects observed is also of interest. These studies should be applied to emerging materials and procedures. Moreover, the use of matrices that allow to evaluate in a long term the potential accumulation of metals in orthodontic patients (i.e. scalp hair) is valuable. The systematic revision concludes the need to perform rigorous randomized clinical trials to explore to what extent the genotoxic damage observed is maintained in the oral mucosa in young target population.

SECOND. A sensitive, reproducible, accurate and robust analytical method has been developed and validated. This method is a useful tool to determine simultaneously the released content of Co, Cr, Cu, Ni, Ti, V and Zr from orthodontic appliances in vivo, in oral mucosa cells of treated patients, by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The method was lineal in a concentration range of 0,5-100 ng/mL. Acceptable LODs and LOQs values were obtained, with recoveries in a range of 84- 111%. Repeatability values and intermediate precision (%RSD) were also satisfactory.

THIRD. The application of the previously validated method in oral mucosa cells from 40 subjects, 20 with an orthodontic treatment and 20 control subjects, has shown significant increases in the concentrations of Co, Cr, Cu and Ni in orthodontic patients, although the concentrations found were much lower than the tolerable daily intakes for Ni and Co, or the upper limits of tolerable intakes established for Cu and Cr (III).

FOURTH. The determination with validated methods by Atomic Absorption Spectrometry (AAS) of Cu, Cr, Fe, Mn and Ni contents in scalp hair of 70 orthodontic patients, in comparison to a control population of 56 subjects, showed a significant increase only for Mn in orthodontic patients in comparison to the control group. Taking into account that the metallic content found was in the range of other previously reported control populations, it is concluded that the accumulation of metallic ions from orthodontic appliances in the selected population does not constitute an important toxic risk.

FIFTH. The biomonitoring study on scalp hair showed an increased metallic content in women in comparison to men. In relation with the age, only increased concentrations of Mn were detected in young subjects (<20 years). Positive correlations were detected between Cu/Mn, Cu/Ni and Fe/Ni and they can be explained by synergisms, probably due to their chemical similarity. This study has allowed to obtain data about metals content in this matrix that can be very useful for comparative purposes. It is concluded that the determination of metallic ions in human scalp hair is a valid method to biomonitorize the release of these elements.

SIXTH. The release of metallic ions, Al, Cu, Cr, Mn, Ti and V in 20 patients with a traditional orthodontic treatment, in comparison to 20 patients treated

additionally with miniscrews, and control subjects, showed the following increasing concentration trend: Cr < Ni < Ti < Cu < Al, whereas V and Co were not detected. A significant increased release of Ni ions in both groups of patients (orthodontic patients and orthodontic + miniscrew patients), and an increase of Cu only in orthodontic patients in comparison to the control group. It was demonstrated a positive correlation in the release of Al/Ti. On the other hand, no correlations were found between the release of metals and different clinical or treatment-related factors such as: gender, presence of miniscrew, type of braces used and their placement.

SEVENTH. The optimization of the alkaline comet assay in oral mucosa cells and its application in patients with orthodontic treatment and orthodontic + miniscrew treatment to evaluate potential genotoxid effect, showed a significant increase of % DNA in tail in the treated groups, in comparison to the control group, although miniscrews do not induce a higher effect. It was observed, although the sample size was small, that the gender does not have any influence on the genotoxicity of the orthodontic appliances.

EIGHTH. Finally, taking into account previous conclusions, the biomonitorization of characteristic metals from orthodontic appliances in oral mucosa cells and scalp hair of patients under treatment is proposed as an adequate strategy to evaluate new orthodontic materials and procedures. Also, the *in vivo* comet assay has shown to be a valuable method to study the induction of genotoxic effects by this kind of materials.