Cytotoxicity Of Orthodontic Temporary Anchorage Devices In Vitro

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CYTOTOXICITY OF ORTHODONTIC TEMPORARY ANCHORAGE DEVICES ON HUMAN PERIODONTAL LIGAMENT FIBROBLASTS *IN VITRO*

by
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ABSTRACT

CYTOTOXICITY OF ORTHODONTIC TEMPORARY ANCHORAGE DEVICES ON HUMAN PERIODONTAL LIGAMENT FIBROBLASTS *IN VITRO*

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Introduction: Cytotoxicity is a major concern in the clinical application of dental materials including Temporary Anchorage Devices (TADs). The purpose of this study was to test the cytotoxicity of four of the commercially available brands of TADs (Aarhus, American Orthodontics; Dual top, RMO; Vector TAS, ORMCO; Unitek TAD, 3M UNITEK).

Materials and Methods: Twenty-four (six from each brand) TADs were individually incubated in complete cell culture medium and shaken at a rate of 1.5 rpm at 37ºC for 30 days to generate the conditioned medium (CM). To test the cytotoxicity, human periodontal ligament fibroblasts (hPDLF) were exposed to the CM for 24 hours. As endpoints, morphological changes were observed along with cell death and damage which were quantified by MTT and LDH assays, followed by statistical analysis of one-way ANOVA with Bonferroni adjustment.

Results: No morphological changes were found in any of the four types of TADs compared to the control cells. LDH assay showed that none of the tested TADs caused significant cell death after CM treatment in contrast to the positive control ($P > 0.05$). No significant intragroup differences were found between any of the four brands of TADs ($P > 0.05$). MTT assay showed similar results as for the LDH assay, except for a marginally significant increase of MTT release found in the TADs from 3M UNITEK compared to the negative control ($P = 0.047$).

Conclusions: According to the ISO10993:5 standards, none of the tested TADs exhibited statistically significant cytotoxicity, suggesting their safe clinical application.

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INTRODUCTION

Temporary Anchorage Devices (TADs) are routinely used as a means of skeletal anchorage in contemporary orthodontics. Their multifaceted use has revolutionized our specialty as we can use them as means for direct or indirect anchorage for various types of orthodontic tooth movements. Historically, pure titanium (cpTi) has been used widely as an implant material because of its excellent biocompatibility. However, cpTi has low fatigue strength and alloying it with aluminum and vanadium to form the titanium alloy Ti–6Al–4V helps in overcoming this disadvantage. The corrosion resistance of the alloy Ti-6Al-4V is lower than of cpTi, giving rise to potential metal ion release. There are complications associated with TAD insertion such as localized inflammation, clinical failure of TADs, osteolysis, cutaneous allergic reactions, remote site accumulation, cytotoxicity, hypersensitivity and carcinogenesis (de Morais *et al.*, 2009). Therefore, from the stand points of either biosafety or potential cause of failure of the clinical application of TADs, it is imperative to study the biocompatibility of TADs. Although many studies have been done on dental implants, the biological effects of TADs on oral cells are poorly understood. The aim of our research project was to explore the biological effects of four brands of commercially available TADs on human periodontal ligament fibroblasts (hPDLF). The null hypothesis was that none of the tested TADs will exhibit cytotoxic effects on hPDLF cells.

REVIEW OF LITERATURE

Anchorage: The backbone of orthodontics

Newton's third law of motion "Every action has an equal and opposite reaction" has been realized as a truth and holds the key to success in orthodontics. In 1923, Louis Ottofy defined it as "the base against which orthodontic force or reaction of orthodontic force is applied" (Ottofy, 1923). It has been defined as "resistance to unwanted tooth movement" by Daskalogiannakis (Daskalogiannakis 2009). Different classifications based on anchorage needs have emerged over the years including *maximum, moderate,* and *minimum* and so called type A, B, and C (Gianelly *et al*., 1971; Marcotte 1990; Burstone 1995).

Need for Skeletal anchorage: Historical development

The need for skeletal anchorage in orthodontics increased with the growing numbers of adult patients seeking orthodontic treatment. In addition, complex treatment goals, patients with missing teeth, non-compliance with extra-oral anchorage all added to the growing need to the skeletal anchorage.

The idea of using bone screws dates back to 1945, when Gains forth and Higley placed vitallium screws in the ascending ramus in dogs for canine retraction (Gainsforth BL and Higley LB 1945). The first clinically reported use in humans came from Creekmore and Eklund in 1983 when they inserted vitallium bone screws in the anterior nasal spine to treat a patient with deep bite. An elastomer was used from the screw to intrude the incisors 10 days after the screw was placed (Creekmore and Eklund, 1983). Soon after, in 1985 Jenner used mini-plates successfully as anchorage (Jenner and

Fitzpatrick, 1985). However, the use of mini-implants was not embraced until 1997, when Kanomi described a mini-implant specifically made for orthodontic use (Kanomi, 1997). Soon after in 1998, Costa *et al.* presented a screw with a bracket like head (Costa *et al.,* 1998). These mini-screws which were temporarily fixed to bone for the purpose of enhancing orthodontic anchorage came to be known as temporary anchorage devices (TADs) and ever since, rapid developments ensued in this area of skeletal anchorage.

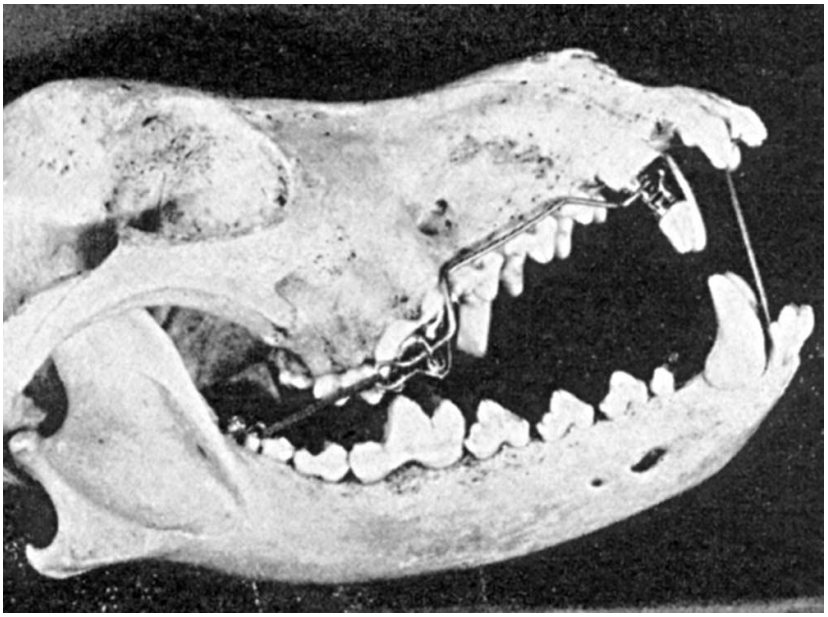

Figure 1.Orthodontic appliance for vitallium screw anchorage. (Redrawn from Gainsforth and Higley, 1945)

Classification of TADs

A. Cope's classification

In 2005, Cope classified TADs as biocompatible and biological in nature. He proposed that ankylosed or dilacerated teeth which are inherently present in the jaws can serve as temporary anchorage devices and are biological TADs which will later be removed. Dental implants and their modifications, fixation screws and wires were

biocompatible temporary anchorage devices.

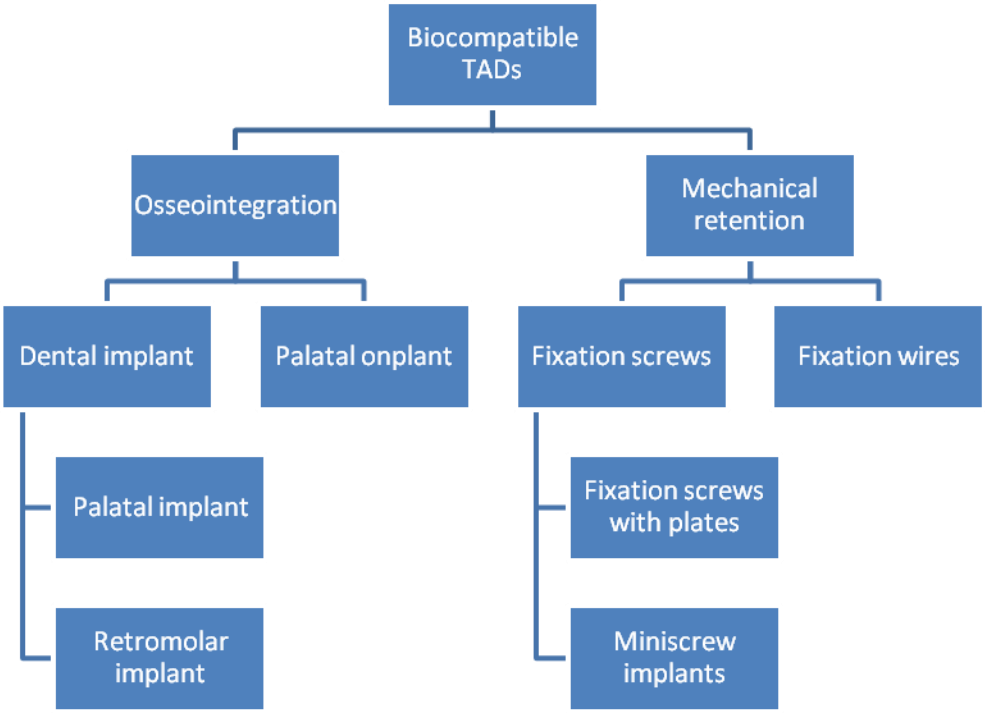

Figure 2.Biocompatible temporary anchorage devices. Redrawn with permission from from Cope JB 2005

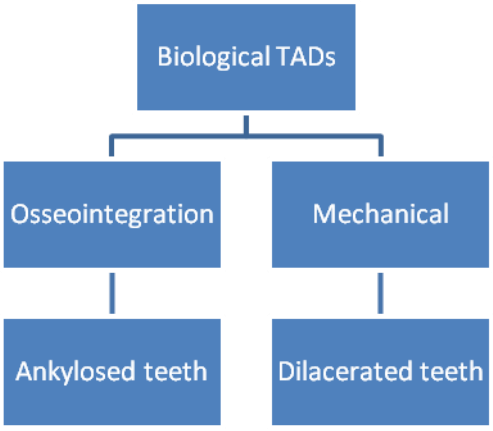

Figure3. Biological temporary anchorage devices. Redrawn with permission from Cope JB 2005

B. Labanauskaite's classification**:**

Dental implants placed for the ultimate purpose of supporting a prosthesis, are not considered temporary anchorage devices since they are not removed and discarded after orthodontic treatment.

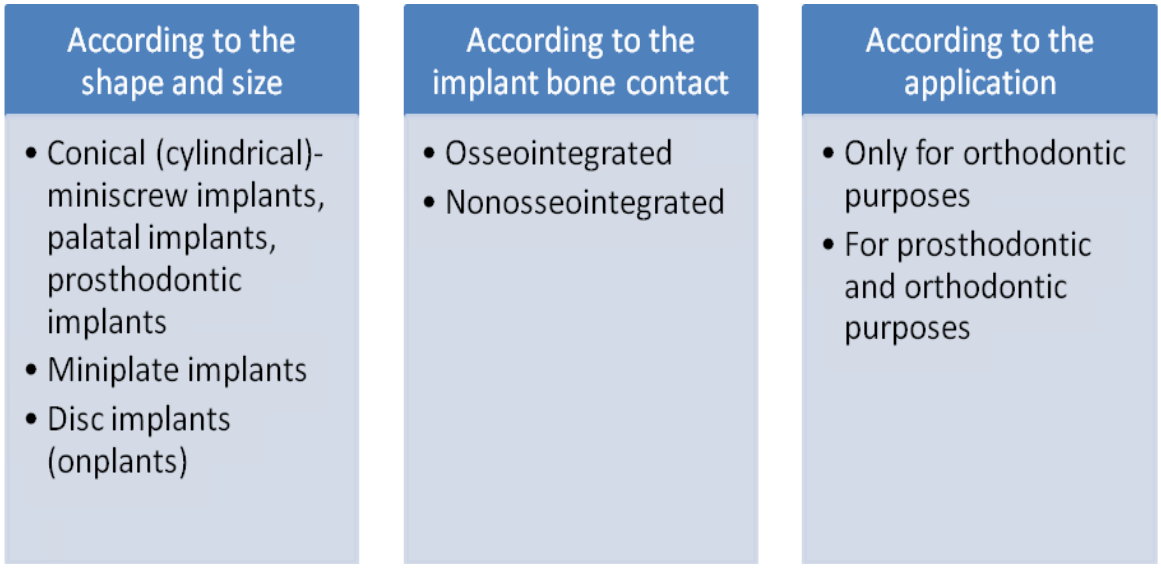

Figure 4. Labanauskaite's classification (Adapted from Labanauskaite B et al 2005)

C. Insertion Modality

- Self -drilling (self-cutting)
- Self – tapping: require pre-drilling at full length

Types of application as anchorage

Direct: Force is applied directly to the tooth /group of teeth from the mini-screw

Indirect: Mini-screw anchors teeth to which the force is applied and prevents movement of these teeth (Papadopoulos MA and Tarawneh F 2007).

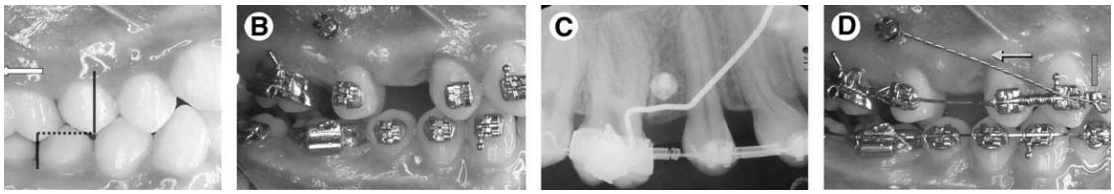

Figure5. Indirect anchorage from a TAD(A) Right buccal with Class II malocclusion. (B) Right buccal with TAD inserted after upper 1st molar distalization. (C) Radiograph showing interproximal location of TAD (D) Upper right canine ligated to the TAD to distalize 1st premolar(From Maino BG et al 2005).

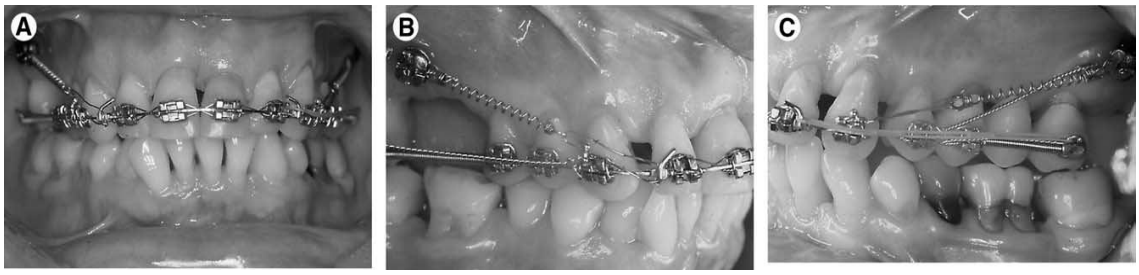

Figure 6.Direct anchorage from TAD to retract anterior teeth. (A) Frontal (B) Right buccal (C) Left buccal (From Maino BG et al 2005).

Indications and Contraindications

TADs are now used for a plethora of orthodontic tooth movements including insufficient number of teeth and/or lack of occlusion in the anchorage unit, extrusion or intrusion of single teeth or units of teeth without antagonists; asymmetric tooth movements, unilateral expansion, asymmetric cant correction; retraction and/or intrusion of anterior teeth, mesial or distal movement of molars, proclination of anterior teeth in cases where no posterior anchoring element is available, space closure in maximum anchorage cases, non-compliant cases. With their smaller sizes, wider applications in tooth movements, simpler surgical placement and immediate loading, TADs have become a mainstay in contemporary orthodontics (Costa *et al.*, 1998; Papadopoulos *et al.*, 2007;

Wahl 2008; Yanosky *et al.*, 2008; Reynders *et al.*, 2009; Melsen 2010; Shirck *et al.*, 2011; Yamaguchi *et al.*, 2012). Contraindications to the use include, but are not limited to, patients with systemic conditions such as metabolic bone diseases, immune suppressive therapy, bisphosphonate medication, poor quality bone tissue, hypersensitivity, radiotherapy in the head and neck damage (Melsen, 2005).

Composition of TADs

Most commercially available orthodontic mini-implants are made of titanium alloys, primarily Ti-6Al-4V (titanium grade IV- V). Although stainless steel screws were marketed initially, the greater biocompatibility of titanium made it a more attractive option (Maino *et al.*, 2005; Reynders *et al.*, 2009; Proffit WR 5th ed). Titanium has the property of oxidizing in the presence of air and aqueous electrolytes to form a passive titanium dioxide film which contributes to its biocompatibility and corrosion resistance (Velasco-Ortega E *et al.*, 2010), but needs to be alloyed to improve its strength and fatigue resistance (Eliades *et al.*, 2009). The titanium alloy is composed of a fusion of two phases, alpha (6% aluminum) and beta (4% vanadium). Both phases in equilibrium contribute towards advantages of mechanical resilience (alpha phase), good formability and fatigue resistance (beta phase), however this leads to a decrease in the corrosion resistance of the Ti alloy in body fluids (Cotrim-Ferreira *et al.*, 2010).

Table1. Atomic percentages of elements in commercially available TADs (Malkoc *et al.*, 2012)

	Atomic percentages								
Brand	C	Al	S	Cr	Fe	Ni	Ti	V	Manufacturer
Mini Ortho implant	6.7	1.34	2.53	18.4	58.1	12.4	-	-	Leone, Italy
Abso Anchor	5.8	8.56	-	0.77	0.84	0.42	83.41	0.18	Dentos, South Korea
MTN	6.2	8.21	-	0.54	0.49	0.51	83.81	0.15	MTN, Turkey
IMTEC Ortho	4.56	8.83	-	0.43	0.46	0.49	84.83	0.32	3M Unitek, Okla
Vector TAS	4.19	8.12	-	0.62	0.47	0.42	86.56	0.13	Ormco, Calif

Components of TADs

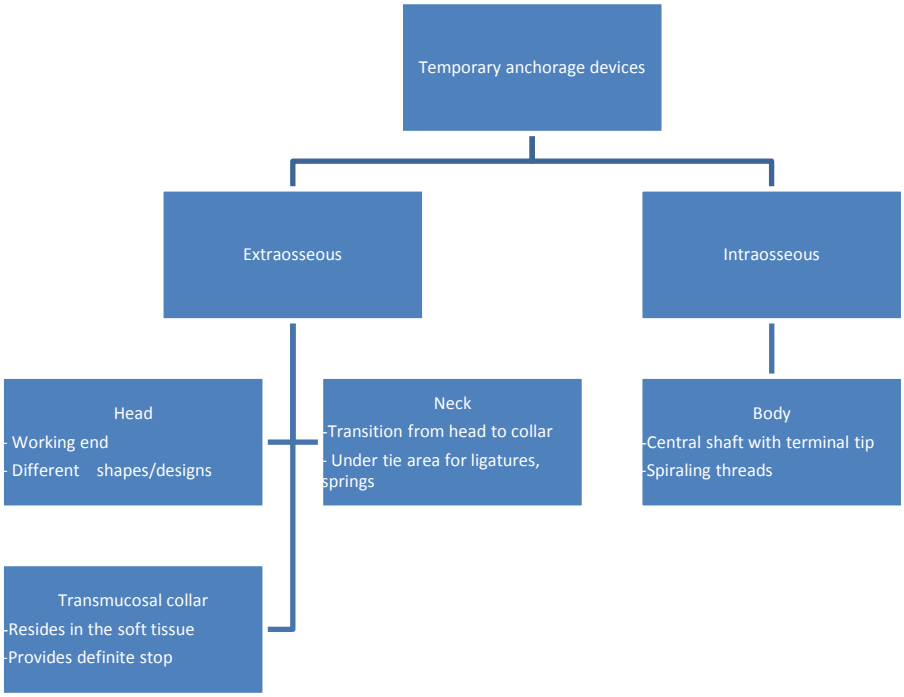

Figure7. Components of TADs

A successful material to be used as for TAD should have good mechanical properties, corrosion resistance and should be biocompatible.

Biocompatibility

Biocompatibility has been defined as "the ability of a material to function in a specific application in the presence of an appropriate host response" by Williams in 1987. This definition rebuked the original philosophy that a material is to be inert in every biological and physicochemical manner to be considered biocompatible. This also helped in defining the basic ideas of biocompatibility and is described below.

a. Interactions at the material–tissue interface affect both the host and the material. Concomitant to the host response to the material, there is a response of the material in the host environment via corrosion, chemical modification, degradation, or other mechanisms. It is a dynamic interaction. It is not static and there are constant influences of aging, local and systemic factors in the host environment and this in turn makes for an ever changing interface where any equilibrium achieved is likely transient and vulnerable to perturbation (Williams, 2008).

b. The reactions at the material–tissue interface are a function of the tissue where the interface is created. The same biomaterial will create a different interface if implanted in the pulp, the bone, the skin. Therefore, favorable material-biological responses in one environment do not assure the same in other environments (Anderson, 2001).

c. Biomaterials are foreign bodies, and biological responses to these materials are characterized by foreign body responses. Avoiding or limiting the foreign body response has been a major goal of material development.

d. Recently, the research in biocompatibility is looking to customize interactions at the material–tissue interface (Ratner, 2001; Ratner, 2004; Bryant 2004). The aim is to modify the surface of a material to limit nonspecific protein absorption, add peptide sequences to encourage native protein or cell interactions, or provide a three-dimensional structure to encourage matrix formation. This aids in developing materials that degrades by design over time, but additionally directs tissue responses via embedded cells, proteins or drugs. The above developments prompted Williams to update his original definition of biocompatibility "ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response to that specific situation, and optimizing the clinically relevant performance of that therapy" (Williams, 2008).

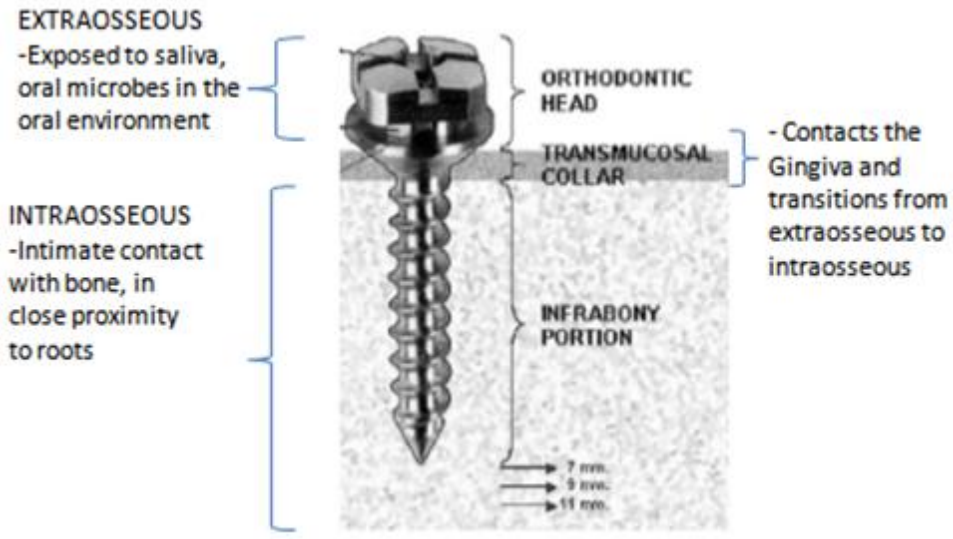

Figure 8.The different host- material interfaces of mini-screws
(Adapted from Maino BG et al 2005).

Host – mini-screw interactions

Placement of the implant in an intraoral site induces several phenomena, including reduction of the pH of the early exudative phases, activation of cells including polymorphonuclear granulocytes and macrophages, and the release of proteins, enzymes, and oxidizing agents that might significantly modify the mini-screw implant surface reactivity. The sequence of events after placement includes the selective adsorption of water, O_2^-, HPO_4^{2-}, and $H2PO_4^-$ and the release of Ti as $Ti(OH)_4$ at the outer oxide layer, with oxide growth at the oxide-metal interface. These interactions result in Ti dioxide films having an outermost layer rich in Ti-hydrogen phosphates, along with increased thickness and a more crystalline and insoluble nature. Calcium-phosphate precipitates can subsequently form on this structure, changing the outer oxide layer to complex Ti and calcium phosphates. Retrieval analyses of mini-screws have shown morphologic alterations such as adsorption of iron, calcium, nitrogen, potassium, phosphorus from the surrounding tissue fluids and these are subsequently calcified with precipitation of calcium and phosphorus (Eliades *et al.,* 2009).

Biocorrosion

Metallic biomaterials implanted in the body undergo an inevitable corrosion process releasing undesirable metal ion/ corrosion products which may or may not be biocompatible. Titanium alloys release titanium {Ti(IV)}, vanadium and aluminum ions (Scales, 1991; Cadosch *et al.,* 2009). Dissolved metal ions then have a propensity to accumulate in the tissue or get transported to remote organs via systemic circulation.

There are different mechanisms for corrosion in the physiological environment and the most common are:

i. Physicochemical corrosion – Contact of metal surfaces with tissue fluids leading to an electrochemical redox reaction (Steinemann, 1996).

ii. Cellular mechanism – Osteoclast precursors have been shown to grown and differentiate towards mature osteoclasts on stainless steel , titanium and aluminum surfaces and directly corrode the metal and take up the metal ions (Cadosch *et al.*, 2009; Cadosch *et al.*, 2010).

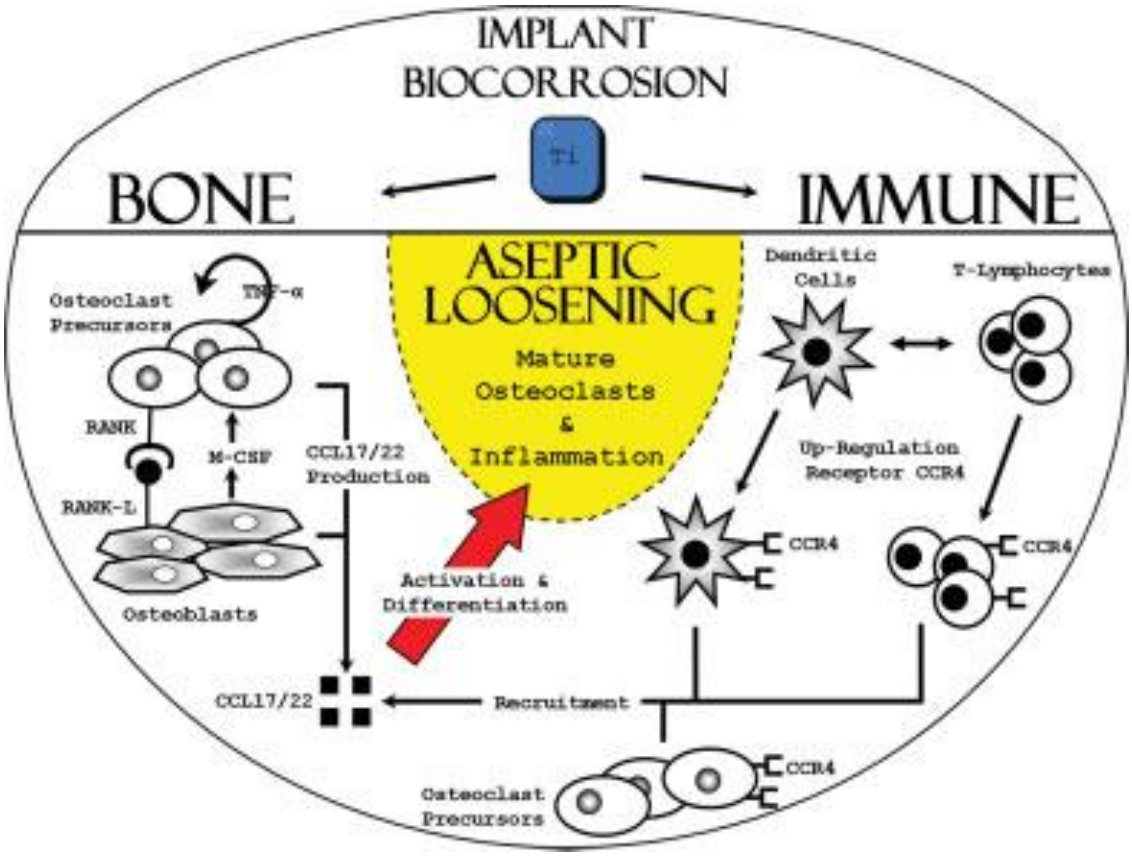

Figure 9. Postulated model involving the effects of titanium ions released by biocorrosion on the immune system and the bone metabolism in the pathophysiological mechanisms of aseptic loosening (Redrawn from Cadosch *et al*., 2010)

Similar processes likely take place *in vivo*. Ti-6Al-4V alloys used in orthopedics for joint replacements have shown to be susceptible to bio-corrosion in the physiological environment of the human body (Scales, 1991; Cadosch *et al.,* 2009; Cadosch *et al.,* 2010). By the same rationale, Ti-6Al-4V alloys used as TADs would be susceptible to bio-corrosion albeit the shorter duration of use as TADs. It has been observed that titanium ions released as part of corrosion may trigger peri-implantitis which further compromises stability (Mouhyi *et al.,* 2009). Inflammation further enhances corrosion by a positive feedback loop (Messer et al 2010) setting up a vicious cycle similar to that seen in the orthopedic aseptic loosening phenomena (Cadosch *et al.,* 2009, 2010). As the osteoclast precursors differentiate and directly corrode titanium surfaces, this in turn affects bone remodeling and hence affects stability. Pits formed around implant surface from corrosion may intensify the corrosive environment, adversely affect its mechanical properties and may hasten fracture of small diameter mini-screws (Gittens *et al.,* 2011). There is some evidence to show that the presence of acidic solutions and fluoride weaken the stable passive protective dioxide layer on Titanium surfaces making it vulnerable to corrosion (Kononen *et al.,* 1995). A recent in-vitro potentiodynamic corrosion study compared the corrosion properties of three miniscrew implants in artificial saliva with and without fluoride. They measured corrosion currents of 2.7-6.0nA which is relatively low and suggested limited ion release in the oral cavity. They reported that exposure to fluoride increased the corrosion current by impacting the passive oxide layer (Knutson and Berzins, 2012). de Morais *et al.* evaluated the systemic levels of metallic ions, specifically the concentrations of titanium, vanadium and aluminum in rabbits with mini-screw implants at 1-week, 4-weeks, and 12-weeks and confirmed that release of these

metals from the mini-implants occurs with diffusion and accumulation in remote organs such as kidneys, liver and lungs. However, despite the tendency of ion release when using the Ti alloy as TADs, the amounts of metals detected were significantly below the average intake of these elements through food and drink and did not reach toxic concentrations (de Morais *et al.*, 2009). Thus there is uncertainty about the role of corrosion and though it may not reach toxic concentrations locally or systemically, it may be a factor in aseptic loosening, in fractures of small diameter mini-screws and may influence the host- mini-screw interactions with pathways unknown as yet.

The success rate of temporary skeletal anchorage devices has been reported to be relatively low compared with dental implants, with failure rates from 9% to 16.4% (Tseng *et al.*, 2006; Lim *et al.*, 2009). To understand failure, we must first gain a better understanding of stability.

Stability

Primary stability is essentially created by mechanical retention of the miniscrew in the bone. It is maximal immediately after insertion but declines concomitantly with bone remodeling that occurs around the screw (Proffit WR, Contemporary Orthodontics 5th ed). This has been linked to the tension-compression state generated at the bone-temporary anchorage device interface (Huja, 2005). *Secondary stability* on the other hand is determined by biologic union of screw to surrounding bone and increases over time with the ensuing remodeling. The net sum of primary and secondary stability is *Clinical stability /Overall stability.* This decline to minimum at 2 weeks post insertion and eventually stabilizes somewhat greater than initial primary stability at about 6 weeks (Proffit WR, Contemporary Orthodontics 5th ed).

Factors related to primary stability include insertion site characteristics such as cortical density and bone quality. TADs inserted in the maxilla have higher success rates than the mandible with the most favorable position relative to the root being the coronal third. With respect to soft tissues, the attached gingiva is favored followed by mucogingival line. The geometric design of the screw which includes the pitch of screw threads, length, diameter, shape, tip form of the miniscrew are all considerations (Holmgren *et al.,* 1998; Motoyoshi *et al.,* 2007; Baek *et al.,* 2008; Kim *et al.,* 2008; Mesa *et al.,* 2008; Cha, 2010; Wang *et al.,* 2010; Zhang *et al.,* 2010; Manni *et al.,* 2011). In regards to the pitch of the screw threads, tighter thread pitch gives greater contact with cortical bone and increases primary stability. The length is variable between 6-10 mm and diameters range from 1.3- 2.0mm. Given that the amount of contact with cortical bone rather than medullary bone is important, screws less than 1.2 mm diameter are avoided and less than 8 mm in length may contribute to decrease in stability as per Crismani *et al.* Larger diameter will show better primary stability under the application of heavy loads but not otherwise (Crismani *et al.,* 2010).Tapered screws have higher placement torques and primary stability, but no difference in removal torque. This may indicate decrease in strain generated during bone remodeling which in turn may affect secondary stability (Chapman, 1996; Migliorati *et al.,* 2011).Thread forming screw compresses the bone around the thread as it advances and provides better bone to screw contact, especially adapted for alveolar bone. Thread cutting screws have a cutting flute on the tip which improves penetration into denser bone, thus performing better in mandibular ramus and buccal shelf, zygomatic buttress and palate (Yerby *et al.,* 2001). The surface roughness of the threaded part of screw- machined vs. roughened is not a

major influence. Preexisting soft tissue inflammation or lack of immaculate hygiene predisposing to inflammation can compromise the stability. Though root proximity is not a key factor in long term stability, it is advisable to avoid contact. The operator technique and inadvertent micromotion have also been implicated in failure of miniscrews. However, immediate or early loading of mini-screws has not shown to be a cause of failure. In addition, longer healing periods have not shown to provide greater stability at forces of up to 200 cN. Maximum insertion torque has emerged as key factor to primary stability of screws (Motoyoshi *et al.*, 2006; Motoyoshi *et al.*, 2007; Lim *et al.*, 2008; Cha, 2010; Suzuki *et al.*, 2011). Despite considering factors of insertion site, meticulous placement and hygiene, geometric design of screws and loading considerations, miniscrews still are prone to loss of stability which makes it essential to analyze host-miniscrew interactions.

Measuring biological responses – In Vitro testing

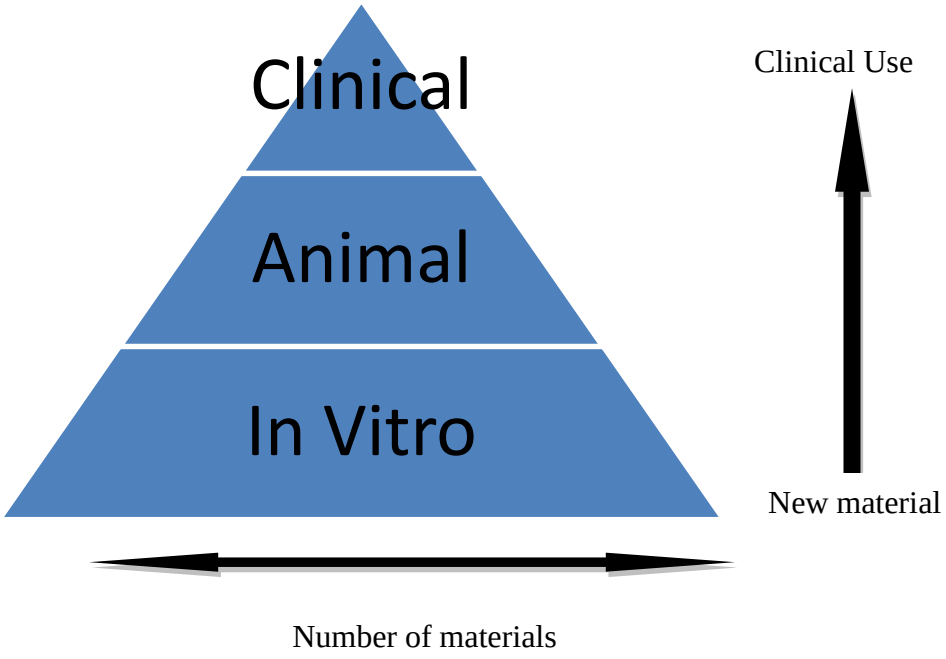

Figure 10.The Classic Paradigm: Biocompatibility assessment of new materials (Adapted from Autian, 1970)

This stepwise testing was proposed by Autian and entailed testing a new material first with *in vitro* tests .This was a screening tool and materials that 'passed' the *in vitro* tests were subsequently tested in animals and clinical trials. Of critical value is the fact that each level of test eliminated unsuitable materials from further testing (Autian, 1970).

By definition, *in vitro* tests occur outside an organism, in a vessel of some sort, using cultures of cells or cell constituents. It is one of the most fundamental ways of testing for biological responses. They are to be differentiated from *ex vivo* tests which use an intact tissue or organ that is maintained for a short time (usually < 24 h) in a culture vessel. In their most sophisticated form, *in vitro* tests use multiple cells, barriers, or special culture conditions to attempt to replicate conditions *in vivo.*

The primary strengths of *in vitro* tests are the ability to control the environment of the cells and their interface with materials and the ability to measure cell response in detail and with precision. Thus they lend themselves well to identify detailed mechanisms of cellular response. In addition, they are faster, less expensive, more reproducible, and more feasible than other types of tests. However, *in vitro* tests may suffer from a lack of relevance to the clinical use of materials and this is an important consideration (Wataha, 2012).

However, adaptation of *in vitro* tests has provided some successes in correlating with the clinical performance of materials. As an example, early *in vitro* tests of ZOE cements predicted acute, severe pulpal toxicity but this was not in echoed in the clinical setting, where it was being used successfully under cavity preparations and *in vivo* pulpal studies verified a relatively low toxicity. The use of a dentin barrier in *in vitro* models reduced the apparent toxicity of ZOE. This created doubts about the reliability of interpreting in vitro test results to the clinical situation and a belief that *in vitro* tests might be more useful if they could be constructed with more appropriate clinical relevance (Wennenburg, 1978; Langleand, 1983).

Principles of In Vitro Cytotoxicity testing: ISO 10993-5:2009(E)

a. Sample and controls

- Test sample- Material / device or extract there of that is subjected to biological /chemical testing or evaluation.
- Positive control – produces reproducible cytotoxic response when tested in accordance with ISO 10993.
- Negative control – Does not produce cytotoxic response and helps in

demonstrating the background response of cells to a stimulus.

- Blank – Extraction vehicle not containing the test sample but retained in the vessel identical to that containing test sample. Aids in assessing possible confounding effects due to extraction vessel, vehicle and extraction process.

b. Extraction vehicles for mammalian cell assays – Culture medium with serum is preferred since it supports cellular growth and is able to extract both ionic and non- ionic compounds.

c. Choice of serum depends on cell type. Serum/ proteins may bind to some extractable and this needs to be considered

d. Extractions should be done in sterile, chemically inert containers using aseptic techniques. For culture medium with serum the temperature should be $(37\pm1)^0$ Celsius to ensure stability of medium and serum.

e. Any processing of extract by means of filtration, centrifuging should be documented in final reporting. Avoid changing the pH of the extract.

f. Cell lines – Established cell lines from recognizable repositories.

Culture medium- Should meet growth requirements of the cells. Antibiotics may be added as long as they do not influence the assays. pH- 7.2-7.4 maintained.

In Vitro Cytotoxicity test procedures

Tests on extracts	Tests by direct contact	Tests by indirect contact
• Extract added to cell suspensions • Allows qualitative and quantitative assessment • Can be performed on original extract or dilution series of the same	• Cell suspension directly exposed to test sample • Allows qualitative and quantitative assessment of cytotoxicity • Undue movement of specimens can cause physical trauma to the cells	• Agar diffusion • Filter diffusion • Qualitative assessment of cytotoxicity • Use of appropriate staining procedures to determine cytotoxicity

Figure 11. *In Vitro* cytotoxicity test procedures(Adapted from ISO 10993-5:2009(E).

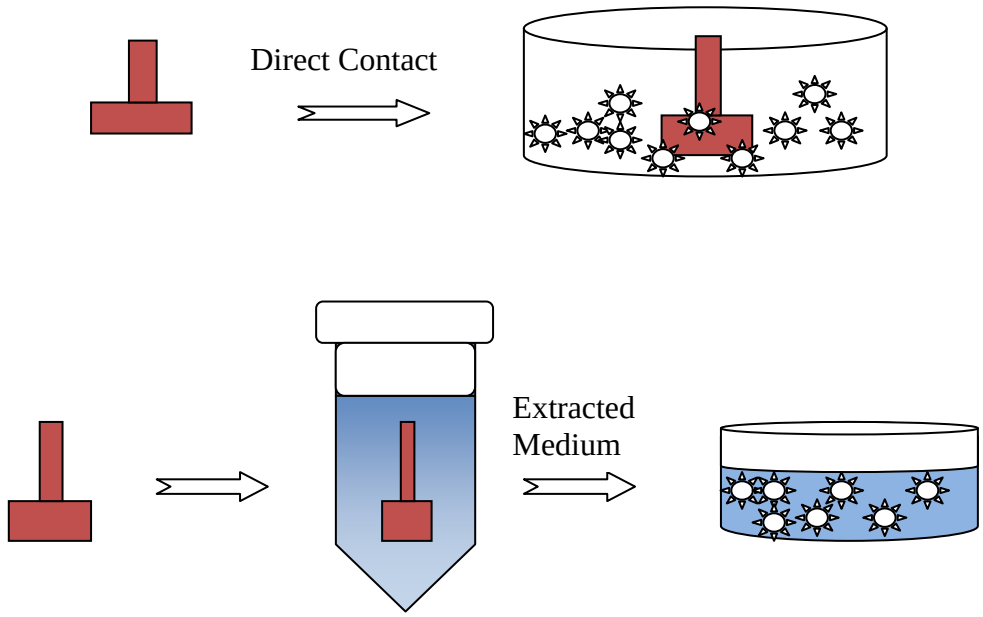

Figure12. Schematic representation of *in vitro* cytotoxicity procedures. (Adapted from ISO 10993-5:2009(E)

Determination of Cytotoxicity ISO 10993-5:2009(E)

A. Qualitative evaluation

- Useful for screening purposes
- Cytochemical staining may be used and cells examined microscopically.
- Assess changes in general morphology, vacuolization, cell lysis, membrane integrity or lack thereof. These may be recorded descriptively or numerically and compared with reference tables provided.

B. Quantitative evaluation

- Preferred means of determination
- Measure cell death, inhibition of cell growth / proliferation or colony formation. These test, utilize number of cells, release of vital dyes, proteins, enzymes or any measurable parameters to quantify the results.
- Reduction of cell viability by more than 30% is considered to be a cytotoxic effect
- Protocols

➢ Neutral red uptake (NRU) uptake cytotoxicity test

➢ Colony formation cytotoxicity test

➢ MTT cytotoxicity test

➢ XTT cytotoxicity test

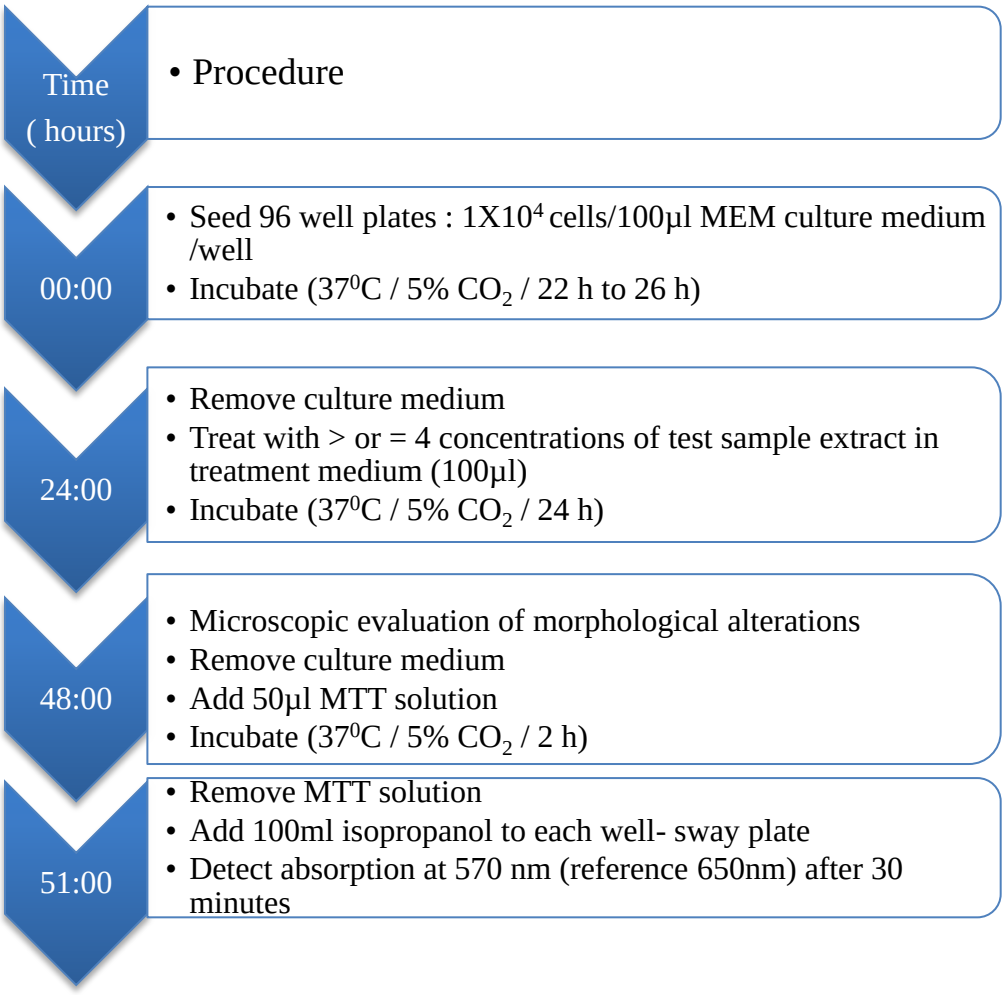

Figure13. MTT cytotoxicity test work flow

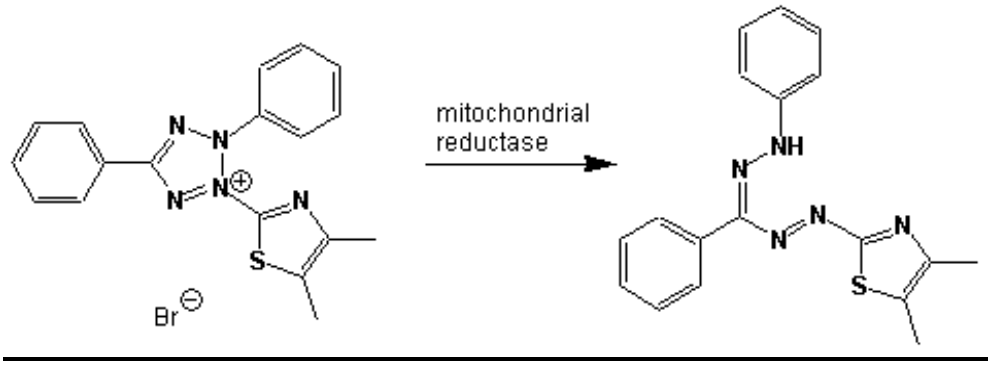

Figure 14. The Principle of MTT colorimetric assay

Cytotoxicity tests of different orthodontic materials from stainless steel wires; latex and non-latex elastics; archwires; esthetic , metallic and nickel free brackets have been undertaken with variable methodologies but in keeping with the basic principles of cytotoxicity testing (David A 2004; Hanson M 2004; Retamoso LB et al 2012; Oh KT 2005).

Cytotoxicity studies relevant to orthodontic mini-screws have used titanium alloy discs (Ti-6Al-4V) subjected to different surface treatments such as nitric oxide passivation and sand blasting and have reported no cytotoxicity to the mouse or human fibroblasts (Velasco- Ortega E *et al.,* 2010). Interestingly, another study using discs observed a transient decrease in cell viability of MC3T3-E1 osteoblasts with the titanium alloy discs at day 4, followed by a partial restoration around 8 days and total restoration after 15 days in the culture. This pattern was noted in the discs irrespective f any specific surface treatment. They concluded that this was a temporary alteration of cell viability to the chemical composition (Citeau *et al.,* 2005). Given that different manufacturers have trace elements added to the generic Ti -6Al-4V alloys along with colored coatings and surface treatments for color coding and passivation treatments, it is clinically relevant to use commercially available mini-screws.

A recent study using commercially available mini-implants, incubated the TADs in Dulbecco's modified eagle's culture medium for 72 hours according to the 2009 version of ISO 10993-5 standards and used a real time cell analyzer to assess cytotoxicity of released bioactive components from TADs on human gingival fibroblasts and mouse osteoblast cells over a period of 190 hours. They observed that the stainless steel containing TADs caused a significant decrease in mouse osteoblast viability. The

remaining TADs were titanium alloys of similar composition – yet two of them showed no adverse effects on MC3T3-E1 cells at 190 hours while the remaining two caused a significant decrease. None of the above tested TADs showed adverse effects on human fibroblasts (Malkoc *et al.*, 2012).

Generally, TADs are likely to be in the oral cavity for 6-8 months and hence if they would release any bioactive components, it may accumulate over time to increase the dosage of bioactive component exposed to adjacent cells.

Objectives of Study

The objective of our study was to test the cytotoxicity of four brands of commercially available TADs. Our null hypothesis was that TADs and their extracts do not cause cytotoxic effects on human periodontal ligament fibroblasts (hPDLF) according to ISO standards. Since TADs remain in the oral cavity for an extended time period, we subjected the hPDLF cells to the conditioned medium collected from the TADs incubated in complete cell culture medium for 30 days. As endpoints, we observed the morphological changes of the cells, and measured the cell viability (via MTT assay) and cell damage (via LDH assay).

MATERIALS AND METHODS

Six of each of the four commercially available brands of TADs (Aarhus, American Orthodontics; Dual Top, RMO; Vector TAS, ORMCO; Unitek TAD, 3M UNITEK) were used in this study. In an effort to standardize the test products, all the chosen TADs had relatively similar length and diameter (Fig 14). All the TADs were used for the cytotoxicity tests directly from their sterile surgical packages, except for the product from American Orthodontics which was autoclaved prior to use due to its unspecified sterile condition. According to the ISO 10993-5 standards, cytotoxicity can be tested by contact (direct) or extraction (indirect) means. Due to the complicated surface topography of TADs, we chose to use the extraction method, that is, to incubate the TADs in cell culture medium for certain time and use the conditioned medium for cytotoxicity testing.

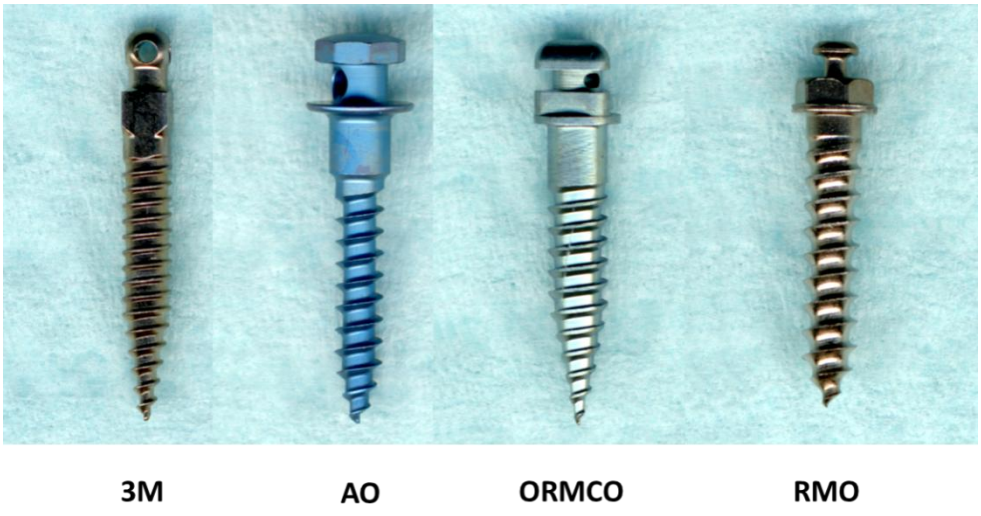

Figure15. Representative TADs from the four brands used for the experiment

In this study, twenty-four TADs of the four kinds (n=6 for each brand) were individually submerged in 8 mL alpha-minimal essential medium (α-MEM) with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (10,000 units of penicillin and 10 mg of Streptomycin in 0.9% NaCl), and sealed in 15mL volume test tubes. To mimic the clinical scenario where the TADs are placed intra-orally and exposed to the flow of saliva, the test tubes were constantly shaken at a speed of 1.5 round per minute (rpm) to simulate physiological salivary flow (Zhou 2010) at 37ºC in incubator for 30 days. The speed of shaking was determined based on our previous study (Zhou 2010). By the end of shaking for 30 days, the conditioned media (CM) were collected for cytotoxicity tests. In addition to the experimental groups, a control group (n=6) was set under the same experimental condition without TADs.

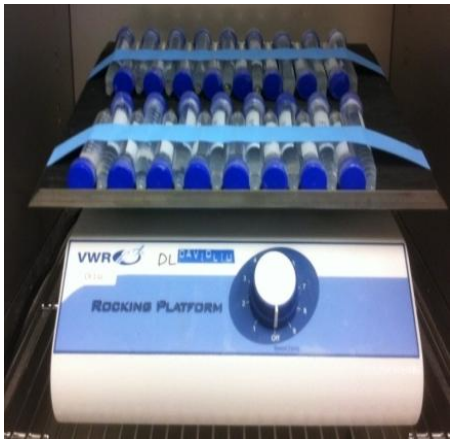

Figure 16. The TADs incubated in cell culture medium on shaker

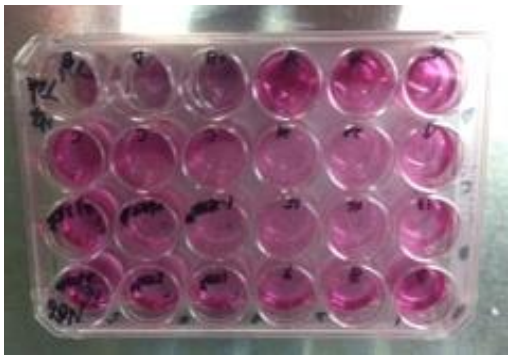

Figure 17. A 24 well plate with seeded cells during the MTT assay

The cell source, hPDLF cells #2630 (Science Cell Research Laboratories, Inc. Carlsbad, CA) were grown in the same type of cell culture medium as used for incubating TADs in a humidified atmosphere of 5% CO_2, 95% air at 37ºC. To test cytotoxicity, the cells ($5X10^4$ cells/ml/well) were seeded in 24-well plates for 24 hours and then treated by the CM for 24 hours. As positive control, 0.1%Triton-100 (Sigma, St. Louis, MO) was used to generate cell damage and death, while the cells treated with the CM without TADs were used as negative control. By the end of 24 hours of treatment, morphological changes as well as cell damage and viability were examined.

The cell shape and size were observed under the microscope (Nikon Eclipse Ti-S, Nikon Instruments Inc, America), and digital images were taken from all the groups in this experiment under the magnification of 20.

LDH Assay

Lactate dehydrogenase (LDH) is an enzyme located in the cytoplasm and is released into surrounding culture medium upon cell damage or lysis. LDH activity in the culture medium can be used as an indicator of cell membrane integrity and hence of cytotoxicity (Haslam, 2000, Wolterbeek, 2005, David, 2004). Quantity of LDH in CM at 24 hours was determined following the assay protocol of Cayman LDH Cytotoxicity

Assay Kit (Life Technologies Corporation, Grand Island, NY). The absorbance was read at 490nm with a plate reader (Bio-Tek power wave XS2, Winooski, VT). Blank LDH levels were subtracted from insult LDH value.

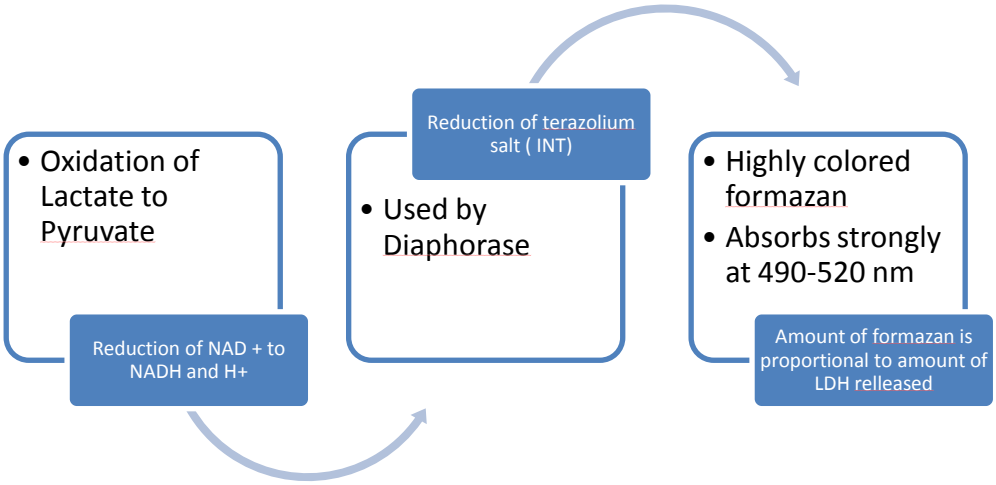

Figure 18. Schematic representation of the principles of LDH assay

Figure19. Micro-plate reader. Bio-Tek power wave XS2, Winooski, VT

MTT assay

The MTT assay is based on the measurement of cell viability via metabolic

activity. Yellow water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5–diphenylytetrazoliumbromid) is metabolically reduced in viable cells to a blue – violet insoluble formazan. The number of viable cells correlates with the color intensity determined by photometric measurements (Scudiero, 1988; Sjögren, 2000). The reduction of MTT is thought to occur mainly in the mitochondria through the action of succinate dehydrogenase, therefore providing a measurement of mitochondrial function. The hPDLF cells damage was thus quantified by measurement of the reduction of MTT to produce dark blue formazan crystals in accordance with the test protocol in MTT kit (Sigma Aldrich, St. Louis, MO). To make the measurement, 75 µl solution MTT was added at 24 hours and after 3 hours of incubation, the medium was removed and the resulting MTT formazan crystals were dissolved by the addition of MTT solvent. The assay of the formation of formazan was performed by measuring the amount of reaction product by absorbance change using the micro-plate reader at a wavelength of 570nm. Each plate included three blank wells containing complete culture medium without cells (ISO 10993-5:2009).

Statistical analysis

All the MTT and LDH assay data (optical densities) were exported in excel file. All the data were expressed as means ± standard deviation (n=6). Statistically, one-way ANOVA was used to determine the difference between all the experimental groups and the control, while Bonferroni adjustment was applied to find out the difference between any two of the four TAD groups. A P value less than 0.05 was considered significant (SPSS, version 11.10, Chicago).

RESULTS

Changes in cell morphology (shape, size and polarity) correspond to the metabolic status of the cells. When cells are injured or undergoing cell death, it can be found that the integrity of the cell membrane is partially or totally lost, and cells usually shrink in size and lose their polarity, ultimately disintegrating. Therefore, these morphological changes can be used to indicate cell damage or death. In our study, the fibroblasts in the negative control and TAD groups appeared to be spindle shaped cells with no distinct abnormal changes while the fibroblasts exposed to 0.1% Triton -100 lost their normal spindle shape and became rounded.

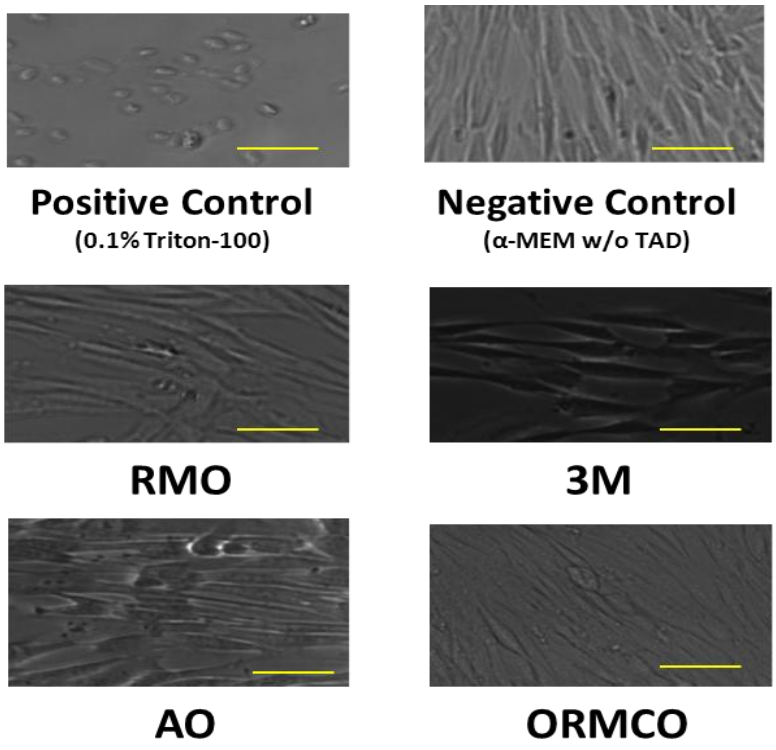

Figure 20. Morphological changes in hPDLF cells subjected to the conditioned media from TADs for 24 hours

Six of each of the commercially available TADs were submerged and shaken with the MEM at a rate of 1.5 rpm for 30 days. We expected the TADs to release metal ions/cytotoxic products over time and then used the extracted conditioned medium to expose the hPDLF and perform tests for cytotoxicity.

The MTT cytotoxicity test (ISO 10993-5:2009) quantitatively measures the cell viability. In accordance with this, our positive control group exposed to 0.1% Triton-100, had the lowest MTT value while the negative control i.e. control conditioned medium without any TAD had the highest MTT levels. There was a significant difference amongst all TADs group along with the negative control as one group in comparison with the positive control ($P = 0.000$). Interestingly, among the four TAD groups, the 3M UNITEK product showed a marginal increase in cytotoxicity (less cell viability) than the negative control ($P = 0.047$)

Table2. Cell viability (MTT) measurements of hPDLF cells subjected to extracts from TADs

Samples	Negative control	Positive control	RMO	ORMCO	AO	3M
1	0.494	0.198	0.326	0.309	0.324	0.332
2	0.449	0.159	0.332	0.315	0.372	0.390
3	0.470	0.121	0.372	0.449	0.360	0.368
4			0.383	0.304	0.285	0.316
5			0.415	0.377	0.476	0.254
6			0.320	0.421	0.532	0.333
Mean ± SD	0.47 ± 0.023	0.16 ± 0.039	0.36 ± 0.038	0.36 ± 0.063	0.39 ± 0.094	0.33 ± 0.050

Table 3. Descriptive statistics for MTT assay results

MTT

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
.0000	3	.159333	.0385011	.0222286	.063691	.254975	.1210	.1980
1.0000	3	.471000	.0225167	.0130000	.415066	.526934	.4490	.4940
2.0000	6	.358000	.0379842	.0155070	.318138	.397862	.3200	.4150
3.0000	6	.362500	.0626985	.0255965	.296702	.428298	.3040	.4490
4.0000	6	.391500	.0939697	.0383630	.292885	.490115	.2850	.5320
5.0000	6	.332167	.0469059	.0191493	.282942	.381391	.2540	.3900
Total	30	.351867	.0933003	.0170342	.317028	.386706	.1210	.5320

Table 4. ANOVA analysis of MTT assay results

MTT

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.166	5	.033	9.290	.000
Within Groups	.086	24	.004		
Total	.252	29			

Table 5. Bonferroni adjustment for MTT assay results

Multiple Comparisons

MTT
Bonferroni

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.0000	1.0000	-.3116667*	.0488764	.000	-.470925	-.152409
	2.0000	-.1986667*	.0423282	.001	-.336588	-.060745
	3.0000	-.2031667*	.0423282	.001	-.341088	-.065245
	4.0000	-.2321667*	.0423282	.000	-.370088	-.094245
	5.0000	-.1728333*	.0423282	.006	-.310755	-.034912
1.0000	.0000	.3116667*	.0488764	.000	.152409	.470925
	2.0000	.1130000	.0423282	.201	-.024921	.250921
	3.0000	.1085000	.0423282	.256	-.029421	.246421
	4.0000	.0795000	.0423282	1.000	-.058421	.217421
	5.0000	.1388333*	.0423282	.047	.000912	.276755
2.0000	.0000	.1986667*	.0423282	.001	.060745	.336588
	1.0000	-.1130000	.0423282	.201	-.250921	.024921
	3.0000	-.0045000	.0345608	1.000	-.117112	.108112
	4.0000	-.0335000	.0345608	1.000	-.146112	.079112
	5.0000	.0258333	.0345608	1.000	-.086779	.138446
3.0000	.0000	.2031667*	.0423282	.001	.065245	.341088
	1.0000	-.1085000	.0423282	.256	-.246421	.029421
	2.0000	.0045000	.0345608	1.000	-.108112	.117112
	4.0000	-.0290000	.0345608	1.000	-.141612	.083612
	5.0000	.0303333	.0345608	1.000	-.082279	.142946
4.0000	.0000	.2321667*	.0423282	.000	.094245	.370088
	1.0000	-.0795000	.0423282	1.000	-.217421	.058421
	2.0000	.0335000	.0345608	1.000	-.079112	.146112
	3.0000	.0290000	.0345608	1.000	-.083612	.141612
	5.0000	.0593333	.0345608	1.000	-.053279	.171946
5.0000	.0000	.1728333*	.0423282	.006	.034912	.310755
	1.0000	-.1388333*	.0423282	.047	-.276755	-.000912
	2.0000	-.0258333	.0345608	1.000	-.138446	.086779
	3.0000	-.0303333	.0345608	1.000	-.142946	.082279
	4.0000	-.0593333	.0345608	1.000	-.171946	.053279

*. The mean difference is significant at the 0.05 level.

Note: 0 – positive control, 1 – negative control, 2 – RMO, 3 – ORMCO, 4 – AO, 5 – 3M

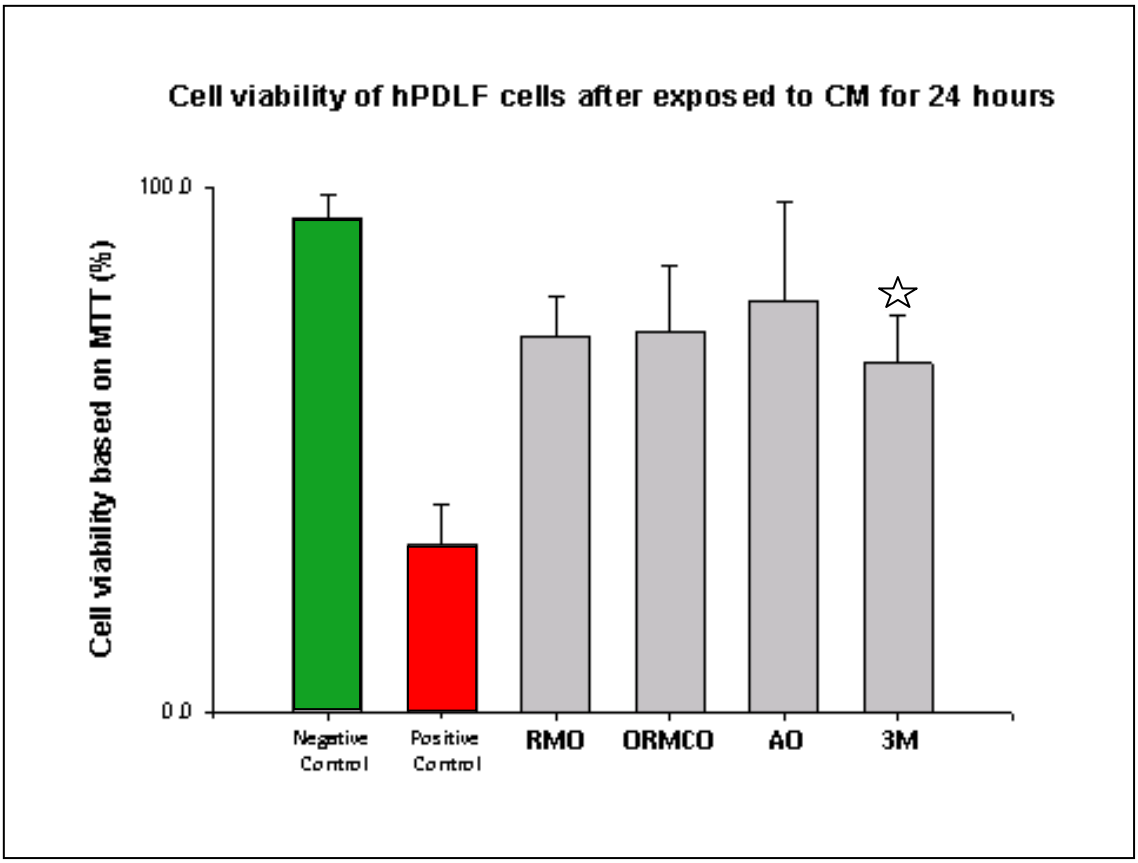

Figure 21.Cell viability (MTT) measurement of the hPDLF cells subjected to the conditioned media from TADs for 24 hours (*P = 0.047 between 3M and negative control).

As one can see, the conditioned media were harvested after 30 days of merging all the 4 brands of TADs in cell culture media. Afterwards, the hPDLF cells were treated with conditioned media from TADs for 24 hours. The cell viabilities were tested using MTT method. The positive control group was treated by Triton-100 (according to the assay kit manufacturer's instructions), showing the lowest MTT value of 0.16 ± 0.039 (n=3) (the lower the MTT value, the less viable cells). Negative control was the control conditioned medium without any TAD, showing the highest value of 0.47 ± 0.023 (n=3). All four brands of TADs together with the negative control showed significant different MTT results in comparison to the positive control (P = 0.000). 3M UNITEK product

showed a marginal increase in cytotoxicity (less cell viability) than the negative control ($P = 0.047$).

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme naturally present in all cells and releases into cell culture supernatant when the cellular plasma membrane is damaged or upon cell lysis (David, 2004; Han, 2011). LDH activity in the culture medium can be used as an indicator of cell membrane integrity and a measurement of cytotoxicity. In our study, the positive control group cells treated by 0.1% Triton-100, showed the highest released LDH concentration (0.4 ± 0.024) and thus high cytotoxicity while the negative control resulted in the lowest LDH level (0.17 ± 0.012) as expected. All four TAD groups together with the negative control showed significantly lower LDH levels than the positive control ($P = 0.000$). There was no significant difference amongst the different brands of TADs ($P > 0.05$).

Table 6. Cell damage (LDH) measurements of hPDLF cells subjected extracts from TADs for 24 hours

Samples	Negative control	Positive control	RMO	ORMCO	AO	3M
1	0.169	0.379	0.228	0.162	0.165	0.167
2	0.164	0.405	0.228	0.154	0.171	0.156
3	0.186	0.427	0.177	0.162	0.184	0.208
4			0.167	0.155	0.165	0.168
5			0.157	0.153	0.154	0.162
6			0.180	0.167	0.161	0.174
Mean ± SD	0.17 ± 0.012	0.40 ± 0.024	0.19 ± 0.031	0.16 ± 0.006	0.17 ± 0.010	0.17 ± 0.018

Table7. Descriptive statistics of LDH assay results

Descriptives

LDH24

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
.0000	3	.403333	.0242710	.0140129	.343041	.463626	.3785	.4270
1.0000	3	.172500	.0115326	.0066583	.143852	.201148	.1635	.1855
2.0000	6	.189083	.0308260	.0125847	.156733	.221433	.1565	.2275
3.0000	6	.158500	.0056833	.0023202	.152536	.164464	.1530	.1670
4.0000	6	.166417	.0099771	.0040731	.155946	.176887	.1540	.1835
5.0000	6	.172333	.0184328	.0075251	.152989	.191677	.1560	.2080
Total	30	.194850	.0734537	.0134108	.167422	.222278	.1530	.4270

Table 8. ANOVA analysis of LDH assay results

ANOVA

LDH24

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.148	5	.030	83.007	.000
Within Groups	.009	24	.000		
Total	.156	29			

Table 9. Bonferroni adjustment of LDH assay results

Multiple Comparisons

LDH24
Bonferroni

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
.0000	1.0000	.2308333*	.0154141	.000	.180608	.281058
	2.0000	.2142500*	.0133490	.000	.170754	.257746
	3.0000	.2448333*	.0133490	.000	.201337	.288330
	4.0000	.2369167*	.0133490	.000	.193420	.280413
	5.0000	.2310000*	.0133490	.000	.187504	.274496
1.0000	.0000	-.2308333*	.0154141	.000	-.281058	-.180608
	2.0000	-.0165833	.0133490	1.000	-.060080	.026913
	3.0000	.0140000	.0133490	1.000	-.029496	.057496
	4.0000	.0060833	.0133490	1.000	-.037413	.049580
	5.0000	.0001667	.0133490	1.000	-.043330	.043663
2.0000	.0000	-.2142500*	.0133490	.000	-.257746	-.170754
	1.0000	.0165833	.0133490	1.000	-.026913	.060080
	3.0000	.0305833	.0108994	.147	-.004931	.066098
	4.0000	.0226667	.0108994	.726	-.012848	.058181
	5.0000	.0167500	.0108994	1.000	-.018764	.052264
3.0000	.0000	-.2448333*	.0133490	.000	-.288330	-.201337
	1.0000	-.0140000	.0133490	1.000	-.057496	.029496
	2.0000	-.0305833	.0108994	.147	-.066098	.004931
	4.0000	-.0079167	.0108994	1.000	-.043431	.027598
	5.0000	-.0138333	.0108994	1.000	-.049348	.021681
4.0000	.0000	-.2369167*	.0133490	.000	-.280413	-.193420
	1.0000	-.0060833	.0133490	1.000	-.049580	.037413
	2.0000	-.0226667	.0108994	.726	-.058181	.012848
	3.0000	.0079167	.0108994	1.000	-.027598	.043431
	5.0000	-.0059167	.0108994	1.000	-.041431	.029598
5.0000	.0000	-.2310000*	.0133490	.000	-.274496	-.187504
	1.0000	-.0001667	.0133490	1.000	-.043663	.043330
	2.0000	-.0167500	.0108994	1.000	-.052264	.018764
	3.0000	.0138333	.0108994	1.000	-.021681	.049348
	4.0000	.0059167	.0108994	1.000	-.029598	.041431

*. The mean difference is significant at the 0.05 level.

Note: 0 – positive control, 1 – negative control, 2 – RMO, 3 – ORMCO, 4 – AO, 5 – 3M

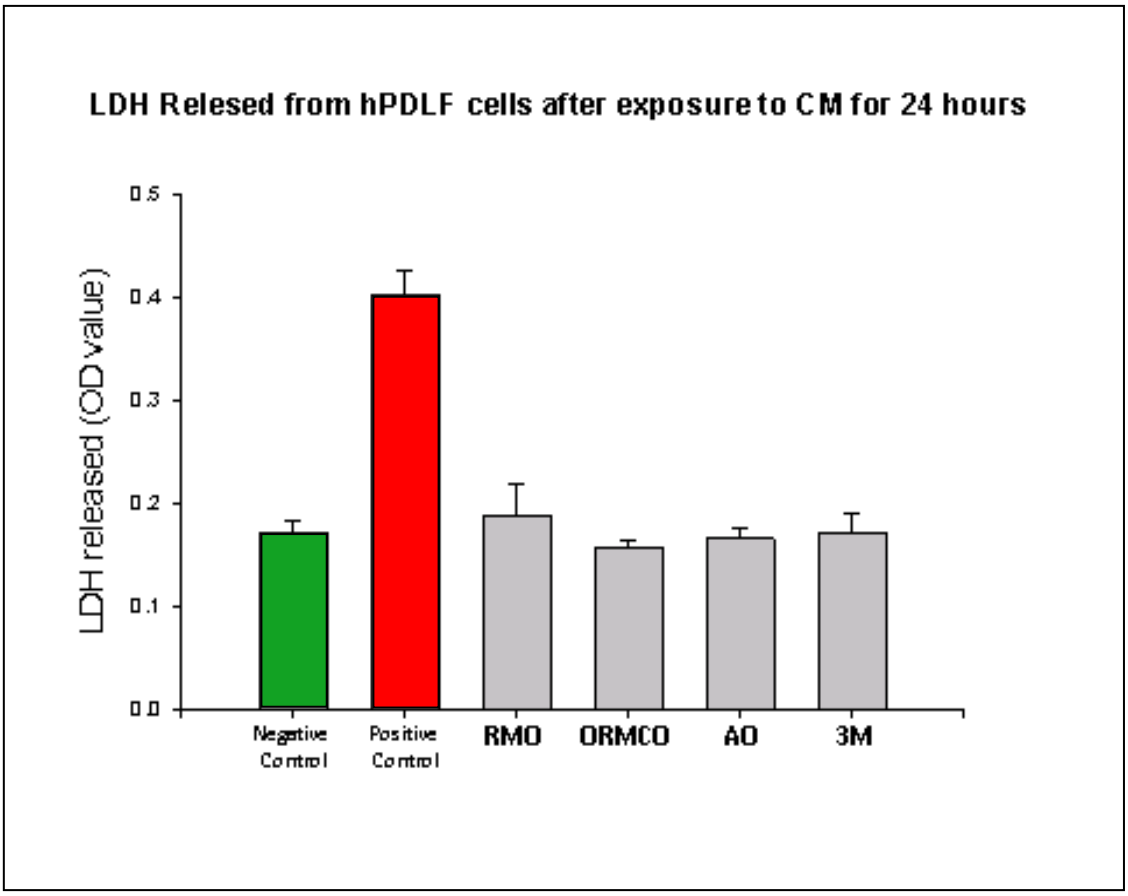

Figure 22: Cell damage (LDH) measurement of the hPDLF cells subjected to the conditioned media from TADs for 24 hours.

As we can see, the conditioned media were harvested after 30 days of merging all the 4 brands of TADs in cell culture media. Afterwards, the hPDLF cells were treated with conditioned media from TADs for 24 hours. The cytotoxicity was tested by measuring LDH release. The positive control group was treated by Triton-100, showing the highest LDH concentration. Negative control was the control conditioned medium without any TAD, showing the lowest LDH level. All four brands of TADs together with the negative control showed significantly different LDH levels from the positive control ($P = 0.000$). There was no significant difference amongst the TADs in relation to LDH assay results ($P > 0.05$).

DISCUSSION

In vitro cytotoxicity tests are advised by the International Standard Organization (ISO) to evaluate acute cytotoxicity of a material (ISO 10993-5:2009) but they can also aid in better understanding of the pathogenicity of sub-acute effects of cytotoxicity. Compared to animal studies and clinical approaches, these tests are generally simple, inexpensive, and can be performed under controlled conditions (Mockers *et al.*, 2002; Samara *et al.*, 2011). Ideally, cytotoxicity tests should be done on the same type of tissue that the tested compounds will be exposed to and efforts should be made to simulate *in vivo* conditions as much as possible.

Cell cultures used for dental material toxicity testing are also advantageous since they are relatively easy to perform, cost effective and easier to control in contrast to animal experiments (Malkoc *et al.*,2012). Since TADs are inserted into alveolar bone in close proximity to periodontal ligament and gingiva, it was decided to use the major cellular component of the human periodontal ligament - the spindle shaped fibroblasts (Fundamentals of Periodontics 2nd ed.) to test the cytotoxicity. According to the ISO standards, cell lines have advantages over primary cells in testing cytotoxicity because they are morphologically and physiologically more homogenous than primary cultures and thus can be utilized reliably and reproducibly (Hernandez –Sierra *et al.*,2011). Therefore, in this study, we chose an established cell line hPDLF #2630 to test the cytotoxicity.

We used commercially available mini-implants rather than using Ti-6Al-4V discs (Watanabe *et al.*, 2004), because it is more clinically relevant to use the product which is used intra-orally. In addition, we submerged the TADs in the conditioned medium for 720

hours (30 days) in a water bath shaker (1.5 rpm) to further simulate normal salivary flow rate and mimic *in vivo* conditions favoring bio-corrosion and release of metal ions over a period of time.

Apart from the MTT assay analysis, no significant differences were seen among the tested TADs for observed cell morphology changes and LDH assay analysis. This is not surprising since all these TADs have similar composition (Ti-6Al-4V), although the exact atomic percentages of the tested TADs were not provided by the manufacturers. An interesting finding in our study was that among the four TAD groups, only the 3M UNITEK TAD showed a slight less cell viability than the negative control ($P = 0.047$) based on the MTT assay analysis. The explanation to this may lie in further evaluating biocorrosion. A recent corrosion study examined mini-screws from 3M Unitek, Ormco and American Orthodontics in artificial saliva and noted a subtle but not significant difference in the passivity of the 3M Unitek mini-screws at potentials above 0.3 V. It was noted that the 3 M Unitek had a comparatively less stable passive layer at potentials above 0.3V (Knutson 2012) and a less stable passive oxide layer typically is associated with greater corrosion rate (Bohni 2005). The authors observed that the silver/grey colored Unitek TADs may suggest a thinner oxide layer in contrast to the American Orthodontics and Ormco mini-screws which were blue and pink colored. TADs may be subjected to different surface treatments accounting for the variability in oxide layer. It is of value to consider however that some manufacturers provide color-coded options to differentiate sizes and locations for use and so not all mini-screws from a particular manufacturer may perform the same (Knutson K, Berzins D 2012). In our study, both 3M and RMO had a similar color but 3M still had a slightly less cell viability based on MTT

analysis. American Orthodontics with the blue color in the study by Knutson *et al.*, had a significantly more noble open circuit potential ($P < 0.05$) compared with the others. Also, acidic solutions and fluorine tends to decrease corrosion resistance by decreasing the stability of the passive layer (Kononen, 1995). Although our TADs were submerged in culture medium instead of artificial saliva, the same medium was used for all test products and hence this additive variable was eliminated in our study design.

Despite similar composition, TADs have shown to cause variable cellular reactions on different cell types in previous studies. In another *in vitro* study, Malkoc *et al.* observed that the same Ti-6Al-4V alloy in MTN and Vector TAS (Ormco) significantly decreased the MC3T3- E1 (mouse osteoblasts) cell viability at 190 hours in contrast to AbsoAnchor (Dentos, South Korea) and IMTEC Ortho (3M Unitek, Europe). The Leone mini-screw (Leone, Italy) which contains stainless steel had the most significant decrease in MC3T3-E1 cell index at 190 hours. None of the TADs had significant adverse effects on human gingival fibroblasts (Malkoc *et al.*, 2012). Velasco-Ortega et al found that Ti-6Al-4V discs pretreated by a nitric acid passivation process were non-toxic to human or mouse fibroblasts. They noted that passivation will lead to a more dense stable oxide layer over the alloy surface and hence increase corrosion resistance (Velasco-Ortega *et al.,* 2010). Interestingly another study done on MC3T3-E1 cells in contact with Titanium alloy discs (Ti-6Al-4V) reported a transient reduction in their cell viability at day 4. This decrease was restored by day 8 and completely eliminated after 15 days (360 hours) in culture .The authors attributed this transient alteration of cell viability to the chemical composition (Citeau *et al.,* 2005). Okazaki *et al.* observed decreased growth ratio of MC3T3- E1 cells around Ti-6Al-4V alloys than in

the presence of pure titanium and suggested that It was because of toxic effects of released Vanadium ions (Okazaki *et al.,* 1998).

To our knowledge, no other published study on mini-screws has studied potential cytotoxicity after submerging TADs in a dynamic cell culture medium for as long. Despite being an *in vitro* study, we have tried to mimic the in vivo conditions by simulating saliva flow and by submerging TADs for 720hrs (30 days) in an attempt to extract the highest possible level of toxic substances from the TADs, which is more stringent than the ISO standards in which 72 hours is the suggested longest period to test the acute effect of toxicity.

Like any experiment, there are areas which could be improved in the study design – namely greater sample size, longer incubation time in medium (3 months of incubation in MEM vs. 30 days), increased duration of cytotoxicity testing (48hrs, 72hrs, up to 190hrs).

To explore the possible relationship between the released metal ions from TADs and the cytotoxicity of the extracts of medium, quantitative evaluation of ion release at each time point should be performed. Due to the time limit and the focus of our study, we did not include a quantitative ion release study as part of this project, which obviously is a deficiency.

As we know, the mechanism of cytotoxicity can be cell necrosis (acute and usually by strong toxins) and apoptosis (a relatively longer time process and can be induced by mild toxins). In this study, we only tested the generic cytotoxicity of the TAD extract on the PDL cells without delineating its mechanism. This is beyond the scope of our study but can be further approached in future studies.

Considering the variables in methodology (discs vs. TADs, end point testing vs. real time cell analysis, incubation and testing duration), different cell origins, it is expected to see variable outcomes. Further standardized cytotoxicity testing using commercially available mini-screws in contact with different cell types (MC3T-E1, hPDLF, gingival fibroblasts) for longer durations may resolve some of the conflicting observations in the reported studies. The release of metal ions from orthodontic TADs might directly affect their biocompatibility. There are no exhaustive data correlating metal ion release from TADs, their biocompatibility and association with failure of orthodontic mini-implants or temporary anchorage devices.

Considering the rising clinical use of TADs in orthodontics, further investigations should be performed to facilitate better understanding of the biological effects of the TADs on oral tissues.

CONCLUSION

Under the conditions of this *in vitro* study, our results show that TADs from the four manufacturers generally exhibited similar insignificant cytotoxicity to human PDL fibroblasts, allowing us to accept the null hypothesis, that is, the TADs are not cytotoxic to hPDLF.

BIBLIOGRAPHY

Anderson JM.Biological responses to materials. Annu Rev Mater Sci 2001;31:81–110.

Autian J. The use of rabbit implants and tissue culture tests for the evaluation of dental materials. Int Dent J 1970;20:481–90.

Baek SH, Kim BM,Kyung SH, Lim JK, Kim YN. Success rate and risk factors associated with mini-implants reinstalled in the maxilla. Angle Orthod 2008;78:895-901.

Bohni H. Localized corrosion of passive metals. In: Revie R W (ed.) Uhlig's corrosion handbook, 2nd ed. 2005Wiley New York pp 173-190.

Brinley C. Pitch and longitudinal fluting effects on the primary stability of mini-screw implants. Angle Orthod 2009;79:1156-61.

Burstone CJ: En masse space closure, in Burstone CJ (ed): Modern Edgewise Mechanics and the Segmented Arch Technique. Glendore,Ormco Corp, 1995, pp 50-60

Cadosch, D., E. Chan, O. P. Gautschi and L. Filgueira.Metal is not inert: role of metal ions released by biocorrosion in aseptic loosening--current concepts. Journal of Biomedical Materials Research Part A 2009;91(4): 1252-1262.

Cadosch, D., M. S. Al-Mushaiqri, O. P. Gautschi, J. Meagher, H. P. Simmen and L.Filgueira. Biocorrosion and uptake of titanium by human osteoclasts. Journal of Biomedical Materials Research Part A 2010;95(4):1004-1010.

Cadosch D, Chan E, Gautschi OP, Filgueira L. Biocorrosion of stainless steel by osteoclasts –In vitro evidence. J Orthop Res 2009;27: 841-846

Cha J. Mini-screw stability evaluated with computerized tomography scanning. Am J Orthod Dentofacial Orthop 2010;137:73-9.

Chapman JR, Harrington RM, Lee KM,Anderson PA, Tencer AF, Kowalski D.Factors affecting the pullout strength of cancellous bone screws. J Biomech Eng 1996;118:391-8.

Citeau A, Guicheux J et al. In vitro biological effects of titanium rough surface obtained by calcium phosphate grid blasting. Biomaterials 2005;Jan 26(2):157-65.

Creekmore TD, Eklund MK. The possibility of skeletal anchorage. Journal of Clin Orthod 1983;17:266-9.

Crismani AG, Berti MH, Cesar AG, Bantleon HP, Burstone CJ. Miniscrews in orthodontic treatment: Review and analysis of published clinical trials. Am J Orthod Dentofacial Orthop 2010;137:108-13.

Cope JB. Semin Orthod 11:3-9 © 2005 TADs- paradigm shift

Costa A, Raffaini M, Melsen B. Mini-screws as orthodontic anchorage: a preliminary report. Int J Adult Orthodon Orthognath Surg 1998;13:201-9.

Cotrim-Ferreira, F. A., C. L. Quaglio, R. P. Peralta, P. E. Carvalho and D. F.Siqueira. Metallographic analysis of the internal microstructure of orthodontic mini-implants. Brazilian Oral Research 2010; 24(4): 438-442.

David A, Lobner D.In vitro cytotoxicity of orthodontic archwires in cortical cell cultures. European Journal of Orthodontics 2004;Aug 26(4):421-6.

Daskalogiannakis J: Glossary of Orthodontic Terms. Leipzig, Quintessence Publishing Co, 2000

de Morais, L. S., G. G. Serra, E. F. Albuquerque Palermo, L. R. Andrade, C. A. Müller, M. A. Meyers and C. N. Elias. Systemic levels of metallic ions released from orthodontic mini-implants. American Journal of Orthodontics and Dentofacial Orthopedics 2009;135(4): 522-529.

Eliades T. Passive film growth on titanium alloys: physicochemical and biologic considerations. Int J Oral Maxillofac Implants 2007;22:743-54.

Eliades T., S. Zinelis, M. A. Papadopoulos and G. Eliades. Characterization of retrieved orthodontic miniscrew implants. American Journal of Orthodontics and Dentofacial Orthopedics 2009;135(1):10.e11-17; discussion 10-11.

Fischer D, Ship JA. Age Ageing 1999; 28:557-561

Gainsforth BL, Higley LB. A study of orthodontic anchorage possibilities in basal bone. Am J Orthod Oral Surg 1945;31:406-16.

Gianelly A, Goldman H: Biologic Basis of Orthodontics. Philadelphia, Lea and Febiger, 1971.

Gittens RA,Olivares-Navarrete R, Tannenbaum R, Boyan BD, Schwartz Z. Electrical implications of corrosion for osseointegration of titanium implants. Journal of Dental Research 2011;90:1389-1397.

Han, X., R. Gelein, N. Corson, P. Wade-Mercer, J. Jiang, P. Biswas, J. N. Finkelstein, A. Elder and G. Oberdörster. Validation of an LDH assay for assessing nanoparticle toxicity. Toxicology 2011;287(1-3): 99-104.

Haslam, G., D. Wyatt and P. A. Kitos Estimating the number of viable animal cells in multi-well cultures based on their lactate dehydrogenase activities. Cytotechnology 2000; 32(1): 63-75.

Hernández-Sierra, J. F., O. Galicia-Cruz, S. A. Angélica, F. Ruiz, M. Pierdant-Pérez and A. J. Pozos-Guillén 2011 In vitro cytotoxicity of silver nanoparticles on human periodontal fibroblasts. Journal of Clinical Pediatric Dentistry 36(1): 37-41.

ISO 10993-5:2009(E).Biological evaluation of medical devices. Part 5: Test for in vitro cytotoxicity.

Holmgren EP, Seckinger RJ, Kilgren LM, Mante F. Evaluating parameters of osseointegrated dental implants using finite element analysis—a two-dimensional comparative study examining the effects of implant diameter, implant shape, and load direction. J Oral Implantol 1998;24:80-8.

Huja SS, Litsky AS, Beck FM, Johnson KA,Larsen PE. Pull-out strength of monocortical screws placed in the maxillae and mandibles of dogs. Am J Orthod Dentofacial Orthop 2005;127:307-13.

Jenner JD, Fitzpatrick BN. Skeletal anchorage utilizing bone plates. Aust Orthod J 1985;9:231-3.

Kanomi R.Mini-implant for orthodontic anchorage. Journal of Clinical Orthodontics 1997; 31(11): 763-767.

Kim JW, Baek SH, Kim TW,Chang YI.Comparison of stability between cylindrical and conical type mini-implants. Angle Orthod 2008;78:692-8.

Knutson KJ, Berzins DW. Corrosion of orthodontic temporary anchorage devices. European Journal of Orthodontics May (Epub) 2012.

Kononen MH, Lavonius ET, Kivilahti JK. SEM observations on stress corossion cracking of commerically pure titanium in a topical flouride solution. Dental Materials1995; 11: 269-272.

Labanauskaite B, Jankauskas G, Vasiliauskas A, Haffar N. Implants for orthodontic anchorage. Metaanalysis. Stomatologija 2005;7:128-32.

Langeland K. Correlation of screening tests to usage tests. J Endod 1978;4:300–3.

Lim S. Insertion torque of orthodontic mini-screws according to changes in shape, diameter and length. Angle Orthod 2008;78:234-40.

Lim HJ, Eun CS, Cho JH, Lee KH, Hwang HS. Factors associated with initial stability of mini-screws for orthodontic treatment. Am J Orthod Dentofacial Orthop 2009;136:236-42.

Luzi C, Verna C, Melsen B. Guidelines for success in placement of orthodontic miniimplants. Journal of Clinical Orthodontics 2009;4339-44.

Maino BG, Paola Mura and John Bednar. Miniscrew Implants:The Spider Screw Anchorage System. Semin Orthod 2005;11:40-46.

Malkoç, S., F. Öztürk, B. Çörekçi, B. S. Bozkurt and S. S.Hakki. Real-time cell analysis of the cytotoxicity of orthodontic mini-implants on human gingival fibroblasts and mouse osteoblasts. American Journal of Orthodontics and Dentofacial Orthopedics 2012;141(4): 419-426.

Manni A, Cozzani M, Tamborrino F, Rinaldis SD, Menini A. Factors influencing the stability of miniscrews. A retrospective study on 300 miniscrews. European Journal of Orthodontics 33(2011)388-395.

Marcotte M: Biomechanics in Orthodontics. Toronto, BD Decker, 1990.

McManus MM, Quian F, Grosland NM, Marshall SD, Southard TE.Effect of miniscrew placement torque on resistance to miniscrew movement under load. Am J Orthod Dentofacial Orthop 2011; 140:e93-8.

Melsen B, The Role of Skeletal Anchorage in Modern Orthodontics. Clinical Orthodontics. Chapter 7, Current Concepts,Goals and Mechanics. Edited by Ashok Karad, 2010.

Melsen B. Mini-implants: Where are we? J Clin Orthod.2005;Sep 39(9):539-47.

Mesa F, Munoz R, Noguerol B, de Dios Luna J, Galindo P, O'Valle F.Multivariate study of factors influencing primary dental implant stability. Clin Oral Implants Res 2008;19:196-200.

Messer RL, Seta F, Mickalonis J, Brown Y, Lewis JB, Wataha JC.Corrosion of phosphate enriched titanium oxide surface dental implants (TiUnite) under in vitro inflammaotory and hyperglycemic conditions. Journal of Biomedical Materials Research Part B: Applied Biomaterials 2010; 92:525-534

Migliorati M, Signori A. Silvestrini Biavati A. Temporary anchorage device stability: an evaluation of thread shape factor. Eur J Orthod 2011 Apr 15 [Epub ahead of print].

Mockers, O., D. Deroze and J. Camps. Cytotoxicity of orthodontic bands, brackets and archwires in vitro. Dental Materials 2002; 18(4): 311-317.

Motoyoshi M, Yoshida T, Ono A, Shi-Mizu N. Effect of cortical bone thickness and implant placement torque on stability of orthodontic mini-implant. Int J Oral Maxillofac Implants 2007; 22: 779-84.

Motoyoshi M, Hirabayashi M, Uemura M, Shimizu N.Recommended placement torque when tightening an orthodontic mini-implant. Clin Oral Implants Res 2006;17:109-14.

Mouhyi J, Dohan Ehrenfest DM, Albrektsson T. The perim-implantitis: implant surfaces , microstructure and physicochemical aspects. Clinical Implant Dentistry and Related Research. 2009

Okazaki et al. Corrosion resistance , mechanical properties, corrosion fatigue strength and cytocompatibility of new Ti alloys without Al and V. Biomaterials 19 ;1998: 1197-1215.

Ottofy L: Standard Dental Dictionary. Chicago, Laird and Lee, Inc, 1923

Papadopoulos MA. and Tarawneh F."The use of miniscrew implants for temporary skeletal anchorage in orthodontics: a comprehensive review." Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007; 103(5): e6-15.

Pickard MB, Dechow P, Rossouw PE, Buschang PH. Effects of miniscrew orientation on implant stability and resistance to failure.Am J Orthod Dentofacial Orthop 2010;137:91-9.

ProffitWR, Fields HW Jr.and Sarver DM. Contemporary Orthodontics, 5th Edition

Ratner BD. Replacing and renewing: synthetic materials, biomimetics, and tissue engineering in implant dentistry. J Dent Educ 2001;65:1340–7.

Ratner BD, Bryant SJ. Biomaterials: where we have been and where we are going. Annu Rev Biomed Eng 2004;6:41–75.

Reynders, R., L. Ronchi and S. Bipat ."Mini-implants in orthodontics: a systematic review of the literature." Am J Orthod Dentofacial Orthop 2009;135(5): 564.e561-519; discussion 564-565.

Samara, A., Y. Sarri, D. Stravopodis, G. N. Tzanetakis, E. G. Kontakiotis and E. Anastasiadou ."A comparative study of the effects of three root-end filling materials on proliferation and adherence of human periodontal ligament fibroblasts." J Endod 2011;37(6): 865-870.

Scales JT. Black staining around titanium alloy prostheses- An orthopedic enigma.J Bone Joint Surg Br 1991;73:534-536.

Scudiero, D. A., R. H. Shoemaker, K. D. Paull, A. Monks, S. Tierney, T. H. Nofziger, M. J. Currens, D. Seniff and M. R. Boyd."Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines." Cancer Res 1988;48(17):4827-4833.

Shirck JM, Firestone AR, Beck FM, Vig KW and Huja SS. "Temporary anchorage device utilization: comparison of usage in orthodontic programs and private practice." Orthodontics(Chic.)2011;12(3): 222-231.

Sjögren, G., G. Sletten and J. E. Dahl."Cytotoxicity of dental alloys, metals, and ceramics assessed by millipore filter, agar overlay, and MTT tests." J Prosthet Dent 2000; 84(2): 229-236.

Steinemann SG. Metal implants and surface reactions. Damage 1996;27(Suppl 3) SC16-SC22.

Su Y, Wilmes B, Honscheid R, Drescher D. Comparison of selftapping and self-drilling orthodontic mini-implants: an animal study of insertion torque and displacement under lateral loading.Int J Oral Maxillofac Implants 2009;24:404-11.

Suzuki EY, Suzuki B. Placement and removal torque values of orthodontic miniscrew implants. Am J Orthod Dentofacial Orthop 2011;139:669-78.

Tseng YC, Hsieh CH, Chen CH, Shen YS, Huang IY, Chen CM. The application of mini-implants for orthodontic anchorage. Int J Oral Maxillofac Surg 2006;35:704-7.

Urban RM, Jacobs JJ, Tomlinson MJ, Gavrilovic J, Black J, Peoc'h M. Dissemintaion of wear particles to the liver, spleen , and abdominal lymph nodes of patients with hip or knee replacement .J Bone Joint Surg Am 2000;82:457-476.

Velasco-Ortega, E., A. Jos, A. M. Cameán, J. Pato-Mourelo and J. J. Segura-Egea."In vitro evaluation of cytotoxicity and genotoxicity of a commercial titanium alloy for dental implantology."Mutat Res 2010;702(1): 17-23.

Wahl, N."Orthodontics in 3 millennia. Chapter 15: Skeletal anchorage." Am J Orthod Dentofacial Orthop 2008;134(5):707-710.

Wang Z, Zhao Z, Xue J, Song J, Deng F, Yang P. Pullout strength of mini-screws placed in anterior mandibles of adult and adolescent dogs: a microcomputed tomographic analysis. Am J Orthod Dentofacial Orthop 2010;137:100-7.

Wataha JC. Predicting clinical biological responses to dental materials.Dental materials 2012;28:23–40.

Watanabe I, Wataha JC, Lockwood PE, Shimizu H, Cai Z, Okabe T. Cytotoxicity of commercial and novel binary titanium alloys with and without a surface-reaction

layer.J Oral Rehabil Feb 2004;31(2):185-9.

Wennberg A, Mjor IA, Hensten-Pettersen A. Biological evaluation of dental restorative materials: a comparison of different test methods. J Biomed Mater Res 1983;17:23–36.

Williams DF. Definitions in biomaterials, Oxford, UK: Elsevier, 1987.

Williams DF. On the mechanisms of biocompatibility.Biomaterials 2008;29:2941–53.

Wilmes B, Su Y, Drescher D. Insertion angle impact on primary stability of orthodontic mini-implants.Angle Orthod 2009;79:609-14.

Wilson TG, Kornman KS. Fundamentalsof Periodontics.Second edition.

Wolterbeek, H. T. and A. J. van der Meer. "Optimization,application,and interpretation of lactate dehydrogenase measurements in microwell determination of cell number and toxicity." Assay Drug Dev Technol 2005;3(6): 675-682.

Yamaguchi,M., T. Inami, K. Ito, K. Kasai and Y. Tanimoto."Mini-implants in the anchorage armamentarium: new paradigms in the orthodontics." Int J Biomater 2012: 394121.

Yanosky, M. R. and J. D. Holmes."Mini-implant temporary anchorage devices: orthodontic applications." Compend Contin Educ Dent 2008;29(1): 12-20; quiz 21, 30.

Yerby S, Scott CC, Evans NJ et al. Effect of cutting flute design on cortical bone screw insertion torque and pullout strength . J Orthop Trauma 15:216-221,2001.

Zhang L, Zhao Z, Lia Y. Osseointegration of orthodontic microscrews after immediate and early loading. Angle Orthod 2010; 80:354-60.

Zhou X, Liu D, You L, Wang L. Estimation of Wall Shear Stress in a Rocking Culture Dish. Journal of Biomechanics.2010, 43: 1598-1602.