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Genotoxicity Effects of Ceramic Coatings Applied on Metallic Substrates using Single Cell Gel Electrophoresis Assay *In Vitro*

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Keywords: Genotoxicity, SCGE, Coatings, Sol-Gel, PVD

Abstract. Sol-Gel coatings are a good choice for protection and bioactivation of metals used as dentistry and standard surgical implant materials. These films should both prevent degradation of the substrates by wear or corrosion, and bioactivate the material for inducing the formation of a hydroxyapatite (HA) rich layer onto the material surface, thereby permitting a natural bonding to living tissues.

The aim of this work was to estimate the clastogenicity *in vitro* by Single Cell Gel Electrophoresis Assay (SCGE) or "comet" assay of coatings of TiN applied by magnetron sputtering and of hybrid layers obtained by Sol-Gel containing glass, glass-ceramic and HA particles on stainless steel AISI 304.

Six test specimens were prepared: AISI 304 Stainless Steel coated with an hybrid silica single film (SF), applied by sol-gel process, AISI 304 SS coated with double film with bioactive glass (DFG), glass-ceramics (DFGC) and HA (DFHA) particles, AISI 304 SS coated with TiN multi films (MFTiN) applied by PVD and bare AISI 304 SS (304SS).

Significantly lower DNA migration ($p>0.005$) was observed in the cells of the cultures corresponding to the samples coated with SF, DFG, DFGC, DFHA and MFTiN respect to the bare 304 SS. The comparison between negative control and the same coated samples did not reveal any statistically significant difference ($p>0.005$) in clastogenicity in vitro evaluated by SCGE.

Introduction

The demand for metallic materials in medical and dental devices is extensive. Metals and alloys are widely used as biomedical materials and are indispensable in the medical field due to their mechanical properties [1, 2]. However, the degradation of the metals implanted in the human body has to be avoided because these devices could exchange ionic species with the organic environment and therefore could affect the signaling and the maturation of cells involved in tissues repair, damaging the structure of the DNA and affecting the cellular viability. In order to improve the surface condition of the metals, several methods are under investigation till date. Coatings are good alternative in order to improve the corrosion resistance of the metals. Sol gel coatings have been widely used for the preparation of films from solutions whit some advantages as such as purity of reactive, simplicity in the preparation, complexity in the compositions, good adherence of coating and low thermal treatments [3-5]. The incorporation of bioactive particles in precursor solutions can be a good choice to combine mechanical resistance and bioactivity in one material improving not only the corrosion resistance of the metal but also its bioactivity [6-8]. The bioactivity of the sol-gel coatings containing bioactive particles has been showed by means of *in vitro* assays without cells [7, 9] and *in vivo* test in Hokaido rats [10]. The evaluation of changes in the structure of the DNA of mamalian cells by effect of the coatings is necessary in order to complement the information about the possibility of the material to be implanted in the human body with the minimum

biological hazard. The aim of this work was to estimate the clastogenicity *in vitro* by Single Cell Gel Electrophoresis Assay (SCGE) of coatings of TiN applied by magnetron sputtering, and of hybrid films obtained by Sol-Gel containing bioactive particles on stainless steel AISI 304.

Materials and methods

Six test specimens were prepared: AISI 304 Stainless Steel coated with an hybrid silica single film (SF), applied by sol-gel process, AISI 304 SS coated with double film with bioactive glass (DFG), glass-ceramics (DFGC) and HA particles (DFHA), AISI 304 SS coated with TiN multi films (MFTiN) applied by PVD and bare AISI 304 SS (304SS).

The sol-gel probes were prepared using hybrids silica sols resulting from acid catalysis in a single stage. Tetraethylorthosilicate (TEOS, Aldrich) and methyltriethoxisilane (MTES, Aldrich) were selected as silica precursors for the sol, alcohol was used as disolvent and HNO_3 and acetic acid were used as catalysts. Three types of bioactive particles were used in this work: Glass, Glass ceramic and Hydroxyapatite. Bioactive glass particles of the CaO-SiO_2-P_2O_5 system were obtained by melting using silica, calcium carbonate (Aldrich) and orthophosphoric acid (Aldrich). The final composition was 57.44% CaO, 35.42% SiO_2 and 7.15% P_2O_5 expressed as molar percentages. The melting of the glass was reached at a temperature of 1600ºC in a period of 2.5 h and quenching was carried out in a container with water at room temperature. Glass-ceramic particles were obtained from the previously described glass, by means of heat treatment (two hours at 1050ºC) until partial crystallization of hydroxyapatite and wollastonite occurred. Hydroxyapatite particles were obtained from the precipitation from calcium nitrate and ammonium phosphate solutions in basic media. The pH of the solutions was controlled with the addition of Ammonium hydroxide (Aldrich). The precipitate obtained was treated at 1050ºC during 1 hour.

To achieve good coatings, is necesary to obtain stable suspensions of the particles in the sol. The suspensions were obtained by adding 10% wt of particles to the silica sol and stirring them with a high shear mixer. Tetrapropyl ammonium hydroxide was used for maintaining glass and glass ceramic particles in suspension and phosphate ester was used by the stability of the hydroxyapatite particles. Two different types of sol-gel coatings were applied: a) Single coatings (SF) from the SiO_2 hybrid sol without particles treated at 450ºC during 30 minutes b) Double coatings consisting on a first coating of the SiO_2 hybrid sol without particles, treated at 450ºC during 30 minutes, followed by a second coating deposited on the top of the first one, of the particle containing suspensions followed by the same heat treatment.

The TiN coatings were applied by PVD using a magnetron sputtering reactor equipped with a Ti target and four magnetrons. Several layers were applied until a thickness of 3 micrometers was obtained.

Peripherical blood samples were obtained by venipunture from six healthy volunteers (three women and three men) 20-40 years old. Cultures were prepared according to the protocol reported by Singh [11]. Each test specimen was placed in a culture tube with 1 ml of blood, 3.5 ml of RPMI-1640 (Sigma), 0.5 ml of Serum Bovine Fetal (SBF), 200 units/ml of penicilin G sodium, 200 µg/ml of streptomycin sulfate, and 0.03 ml phytohematoglutinin. As negative control (C-), the cultures were performed with medium without test specimen, and as positive control (C+), cultures with medium and 0.05 ml of Mitomicin C were prepared. The samples this way obtained were maintained at 37°C in a 5% CO_2 atmosphere during 48 hours. Then, the cultures were centrifuged at 2000 rpm (10 min) and supernatant was removed. Finally, the cells were resuspended in PBS, and the peripherical blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation.

For all experiments the SCGE assay was used to measure DNA damage in individual cell using the alkaline protocol [11]. Cell suspension (10 µl) was mixed with 90 µl of low melting point agarose (Sigma) and extended it on a slide previously coated with 100 µl of 0.5% normal melting point agarose. After gelling at 4°C, the cells were lysed for 60 minutes in a solution of 2.5M NaCl, 100 mM EDTA, 10 mM Tris, 10 % DMSO and 1% Triton X-100, at pH 10 and 4°C. The slides

were washed 3 times in distilled water for 5 minutes and placed them on a horizontal electrophoresis unit, containing fresh electrophoresis buffer (300mM NaOH, 1mM EDTA, pH 13). After 30 minutes of unwinding at 4°C, electrophoresis was performed for 25 minutes (25 V, 300mA). The slides were washed 3 times in 400 mM Tris buffer, pH 7.5 and stained with ethidium bromide (2 μg/ml, 10 min). One hundred cells from each sample were selected randomly and analized by free Comet Score Software. Observations were made at magnification of X200 using a epifluorescent microscope (Zeiss) coupled with CCD camera. All steps were conducted in dark to prevent additional DNA damage.

Cells with damaged DNA displayed high migration of DNA fragments from the nucleus, forming a tail in comet form. Most important parameters used for analyzing clastogenicity in the comet assay were [12]:

- DNA head (DNAH), sum of intensities of all points of the head.
- DNA tail (DNAT), sum of intensities of all points of the tail.
- Percent tail DNA (%DNAT) = 100DNAT/(DNAH+DNAT)
- Tail moment (TM), the product of the tail length and percent tail DNA, TM=TLx(%DNAT)
- Olive tail moment (OTM), the product of the distance (in x direction) between the center of gravity of the head (CGH) and the center of gravity of the tail (CGT) and percent tail DNA, OTM=(CGTx-CGHx)/%DNAT.

Larger values of %DNAT, TM and OTM are related with highly broken DNA strand induced by the interactions between the samples tested and the cultured cells.

Results and Discussion

In the fig. 1A, some comets corresponding to the culture containing the bare AISI 304 SS are showed. The DNA structure has suffered damage in the single strand in comparison with the negative control showed in the fig. 1B.

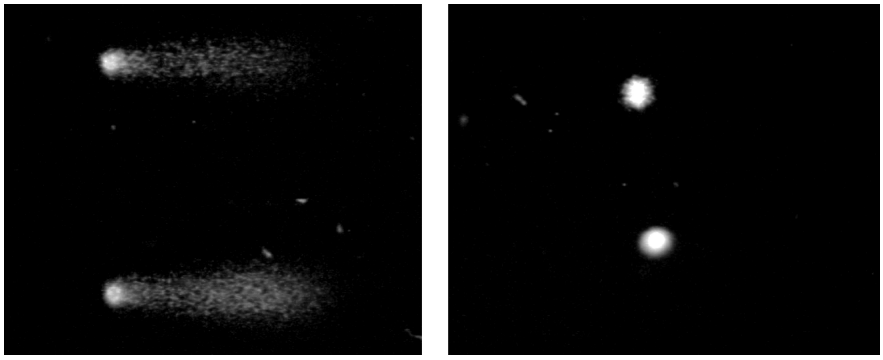
(A)  (B)

Fig.1. Images of PBMC after SGCE assay. (A) Showed DNA with increased single strand broken induced by the presence of bare 304 SS and (B) DNA negative control without single strand broken.

In the fig. 2, the comparison between the OTM medians of the evaluated samples corresponding to the six patients and the positive and negative controls is showed. No statistically significant difference was observed between the OTM of the positive control and the bare AISI 304SS. This could be explained by the metallic ions release from the substrate, which are capable of producing hydroxyl radicals and inducing DNA damage via Fenton and Haber-Weiss-type reactions under physiological conditions [13]. The comparison between cells corresponding to negative control and the coated samples did not reveal any statistically significant difference ($p>0.05$). Thus, the protector effect of the coatings on the substrates is showed, diminishing the release of ions to the medium. The coating containing hydroxyapatite particles showed lower values of OTM indicating less DNA damaged. Some metallic ions adsorb onto calcium hydroxyapatite crystals (HAP) and severely inhibit their dissolution process, when present in concentrations less than 1 microM [14]. This effect probably blocks the hidroxyl radicals production via Fenton and Haber-Weiss-type

reactions under physiological conditions, avoiding the oxidative DNA damage. The explanation of this effect should be studied in future researches. The values of %DNAT and TM showed similar tendencies (data no showed).

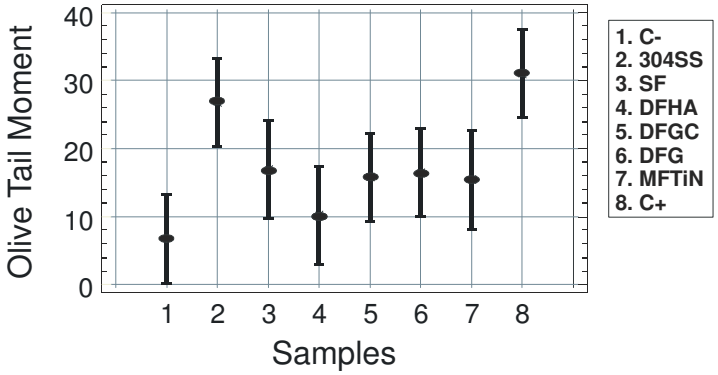


Fig 2. Comparison between the OTM medians of the evaluated samples corresponding to the six patients and the positive and negative controls

Acknowledgement

This study was supported by the DIME (Universidad Nacional de Colombia - Sede Medellín) and Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología –Colciencias- Grant. 1118-12-13724.

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