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In vitro evaluation of cytotoxicity and genotoxicity of a commercial titanium alloy for dental implantology

Eugenio Velasco-Ortega[a,*], Angeles Jos[b], Ana M. Cameán[b], Jesús Pato-Mourelo[a], Juan J. Segura-Egea[a]

[a] Department of Stomatology, School of Dentistry, University of Seville, C/ Avicena s/n, 41009 Seville, Spain
[b] Area of Toxicology, Faculty of Pharmacy, University of Seville, Seville, Spain

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ABSTRACT

Titanium and its alloys have many applications in dentistry, being used in orthodontics, endodontics, prosthetics and implantology. But the use in the biomedical field depends on its biocompatibility, as the Council Directive 93/42/EEC of 14 June 1993 concerning medical devices has established. The aim of this study was to investigate the cytotoxicity and genotoxicity of a commercial titanium/aluminium/vanadium alloy (Ti–6Al–4V) developed by an innovative sand-blast process with aluminium oxide, and nitric-acid passivation. This procedure created a material with an average surface roughness of $1.73 \pm 0.16\ \mu m$ with applications in dental implants. International Organization for Standardization (ISO) procedures 7405:2008 and 10993-5:2009 were used to perform the cytotoxicity tests, and bacterial and cell-mutation assays to evaluate genotoxicity. The results show that this titanium alloy (Ti–6Al–4V) was neither cytotoxic nor genotoxic in any of the tests performed.

It can be concluded that this new Ti–6Al–4V material with the roughness characteristics specified shows good biocompatibility and can be considered of choice in dental implantology.

1. Introduction

The use of titanium and titanium alloys for medical and dental applications has increased considerably in recent years. Historically, titanium has been used extensively in aerospace, aeronautical and marine applications because of its physical and mechanical properties [1]. But these features also make it desirable as a material for implants and prostheses. The strength and rigidity of titanium are comparable to those of other noble or high noble alloys commonly used in dentistry [2], and titanium's ductility, when chemically pure, is similar to that of many dental alloys. Titanium also can be alloyed with other metals, such as aluminium, vanadium or iron, to modify its mechanical properties [3].

In dentistry, titanium and its alloys have many applications in orthodontics, endodontics, prosthetics and implantology. Thus, they are applied to dental products such as endodontic files, crowns, inlays, bridges, etc., as well as dental implants. An increase in the use of titanium in dental applications has been observed, because of its biocompatibility, corrosion resistance, and good physical properties [4].

In this sense, titanium possesses high tissue compatibility, histologically [5,6]. In fact, many previous studies on its electrochemical properties [7], elution into immersion solutions [8,9] or surrounding tissues [10,11], confirmed that titanium has higher resistance to corrosion than other metals. A cytological experiment has shown that titanium has no effect on the distribution or activity of murine macrophages [12]. It has also been shown that titanium has no toxic effects on human fibroblasts [13], and there were no abnormal findings such as inflammatory response in the tissues around the implanted titanium in humans or in some animals [14,6]. For all these reasons titanium and its alloys are currently the most widely used bone-implant materials [15]. Clinical success has been achieved not only because of mechanical strength or excellent biocompatibility of titanium alloys, but also because of other characteristics such as surface properties, especially surface roughness [16]. However, there are some authors who have reported incompatibility or hypersensitivity reactions [17].

New dental materials for clinical use are considered medical devices and have to meet stringent safety and efficacy requirements. Regarding safety issues, the Council Directive 93/42 EEC of 14 June 1993 [18] concerning medical devices, amended by the Directive 2007/47/EC [19] relating to the active, implantable medical devices, is the legal basis for the market launch of dental materials within the European Economic Area and thus also regulates the field of biocompatibility. As it is stated in Annex I, "the devices must be designed and manufactured in such a way that, when used under the conditions and for the purposes intended, they will not compromise the clinical condition or the safety of

* Corresponding author.
E-mail address: evelasco@us.es (E. Velasco-Ortega).

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patients, or the safety and health of users". Also, it is established that "particular attention must be paid to the choice of materials used, particularly as regards toxicity". Furthermore, additional legal regulations of the EU, such as REACH [20], have to be considered for market launches of medical devices [21].

Although tissue reactions to an implant are strongly dependent upon the effects of load and stability of an implanted prosthesis, factors such as design and surface topology play a part [22]. Moreover, the effect of pre-treatment and composition on the cytotoxicity of dental alloys and metals is under investigation [23]. Surface-roughness modulates the osseointegration of dental titanium implants, and diverse processes applied to provide a roughened surface (blasting with silica, aluminium-oxide particles, etc.) may cause the release of cytotoxic aluminium ions into the peri-implant tissue [16]. Despite many studies assessing the cellular response towards implant materials with different surfaces that are commercially available [24], as far as we know there are only two records regarding assessment of the genotoxicity of titanium-disk surfaces [25,26].

In view of the above-mentioned data, the aim of this study was to investigate the biocompatibility *in vitro* of a new prototype commercial titanium-alloy material obtained for an industrial process, which includes blasting with aluminium-oxide particles and nitric-oxide passivation for its future application for dental implants. The cytotoxicity was assessed according to procedures 7405:2008 [27] and 10993-5:2009 [28] of the International Organization for Standardization (ISO), in mouse and human fibroblasts, respectively. Mutagenicity tests used in this study included bacterial and mammalian cell-mutation assays.

2. Materials and methods

2.1. Materials and supplies

The material studied was a commercial titanium/aluminium/vanadium alloy (Ti–6Al–4V, ASTM grade 5) provided by Galimplant S.L. (Sarria, Spain). Discs of machined titanium alloy (5-mm diameter × 1-mm height) with an original surface-roughness $R_a = 0.25\ \mu m$ were obtained following the ISO-5832-3:1996 guideline [29]. They were automatically sand-blasted with aluminium oxide (granulometry of 50–150 μm) at 5-atm pressure in horizontal and 6-atm pressure in angled direction for 30 min. Then a nitric-acid passivation process was performed for 20 min at room temperature, followed by a final wash with distilled water in an ultrasonic bath. Passivation treatments provide a controlled and uniformly oxidized surface state. The passivation leads to a dense and stable oxide film and improves corrosion resistance (decreases ion release). This procedure involves etching with nitric acid. The advantages of this method include an increase in the total surface area of the implant. In addition, it is important that the passivation procedures following sand-blasting remove any particle remnants (especially in the case of alumina or silica) (Fig. 1) [30].

The roughness parameters were determined by use of an optical profilometer Zygo NewView 7300 equipment (ZygoLOT GMBH, Darmstadt, Germany). After passivation with nitric acid the surface-roughness characteristics of the titanium alloy were as follows: average roughness $R_a = 1.73 \pm 0.16\ \mu m$, which is the arithmetic mean of the average variation on the roughness profiles, $R_z = 5.31 \pm 1.02\ \mu m$, which averages the highest point and lowest point over five cutoffs, and $R_{max} = 9.78 \pm 3.04\ \mu m$, i.e. the distance between the highest peak and the lowest valley. Samples were maintained at room temperature until analysis. Culture media, sera and cell-culture reagents were obtained from Gibco (Invitrogen, Spain).

2.2. Cytotoxicity tests

Citotoxicity of the titanium alloy was examined with the agar-diffusion method and its variant using a filter-diffusion model, according to the procedures specified in ISO 7405:2008 [27] and ISO 10993-5:2009 [28]. A negative control of high-density polyethylene (HDPE) and a positive control of polyvinyl chloride (PVC) with organic additives as discs 5 mm × 1 mm were also assayed. They were sterilized by ethanol immersion and with ultraviolet light. Samples were assayed directly or after extraction with cell-culture medium as described in guideline ISO 10993-10:2002 [31].

2.2.1. Cytotoxicity test according to guideline ISO 7405:2008

Mouse fibroblasts, a clone of strain L (NCTC clone 929, ATCC N° CCL-1, American Type Culture Collection, Rockville, MD), were routinely grown in Eagle's minimum essential medium (MEM) supplemented with 10% horse serum, 1% penicillin/streptomycin and 1% L-glutamine and maintained at 37 ± 2 °C in a humidified atmosphere of 5% CO_2 in air.

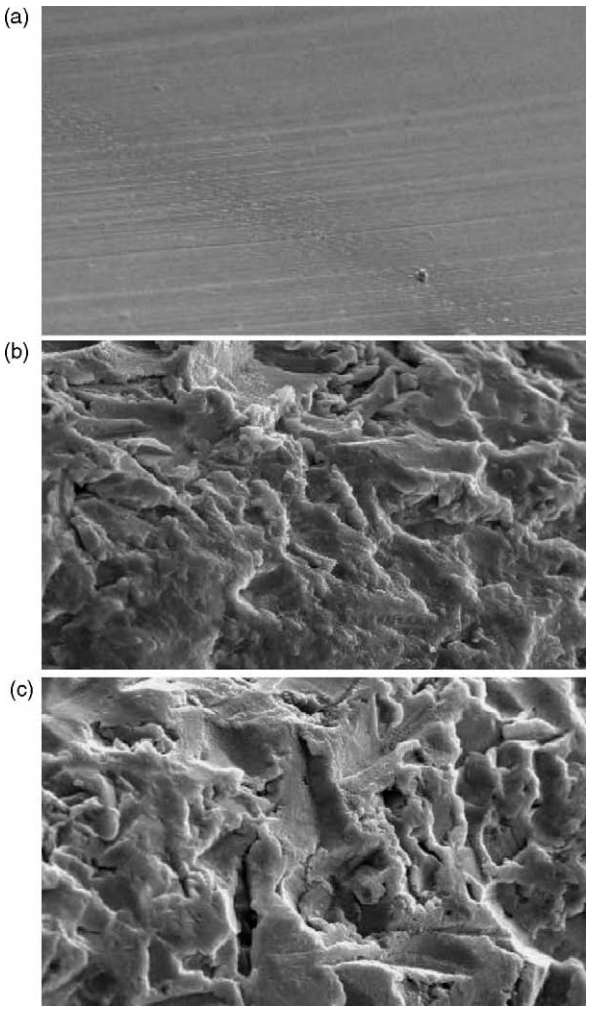

Fig. 1. SEM micrographs showing the surface roughness of the Ti–6Al–4V material: (a) as machined discs; (b) discs sand-blasted with aluminium oxide; and (C) sand-blasted discs after passivation with nitric oxide. Original magnification: 500×, 1000× and 1000×, respectively.

For the *agar-diffusion method* 10 mL of a 2.5×10^5 cell suspension were seeded in 100-mm Petri dishes and incubated at 37 °C and 5% CO_2 for 24 h. The medium was replaced with 10 mL freshly prepared agar/nutrient medium containing 20% serum. Ten mL neutral-red (Sigma, Madrid, Spain) solution was placed on the agar surface for 20 min in the dark. Excess dye was then removed, and the disk samples (titanium material, positive and negative controls) were placed on the agar surface and dishes were incubated for 24 h (37 °C, 5% CO_2). Thereafter, the cultures were examined under a microscope by one examiner experienced in the use of this evaluation technique. The identity of the specimens was unknown to the examiner. The decolorized zones and cell lysis around and/or under the specimens were evaluated using an inverted microscope Olympus CK2-TR (Spain) according to the established criteria (ISO 7405:2008) after 24 h. Three specimens of each material were studied, and each test was repeated twice using the same test specimens. The decolorized zones were scored as follows: 0 = no decolorization detectable; 1 = decolorization only under the specimen; 2 = zone not greater than 5 mm from the specimen; 3 = zone not greater than 10 mm from the specimen; 4 = zone greater than 10 mm from the specimen; 5 = the total culture is decolorized. Cell lysis, defined as loss of cell-membrane integrity that is visible under a light microscope, was scored as follows: 0 = no cell lysis detectable; 1 = less than 20% cell lysis; 2 = 20–40% cell lysis; 3 = 40–60% cell lysis; 4 = 60–80% cell lysis; 5 = more than 80% cell lysis. For each specimen, one score was given, and the median score value for all parallels from each specimen was calculated for both the decolorization zone and the lysis zone. The cytotoxicity was classified as follows: 0–0.5 = non-cytotoxic; 0.6–1.9 = mildly cytotoxic; 2.0–3.9 = moderately cytotoxic; 4.0–5.0 = markedly cytotoxic. The median (instead of the mean) was calculated to describe the central tendency of the scores, because the results are expressed as an index in a ranking scale [23].

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For the *filter-method variant*, 6 mL of a 2.5×10^5 cell suspension were seeded in 50-mm Petri dishes with cellulose acetate filters. After 24 h incubation, cell-culture medium was discarded and filters were washed with phosphate-buffered saline (PBS) and inverted on agar/nutrient medium in new Petri dishes (5 mL/dish). Three borosilicate micro-glass discs were placed per filter, where 0.1 mL of the extracts of the test specimens (sample, negative and positive controls) were added. Samples extracts were obtained following the method described in the guideline ISO 10993-10:2002 [31]. Cell number per filter was obtained by means of flow cytometry (BD FACSCalibur™ cytometer), with 7-amino-actinomycin D staining.

2.2.2. Cytotoxicity test according to guideline ISO 10993-5:2009

Human fibroblasts MRC-5 cells (ATCC N° CCL-171) were maintained in a similar way as previously described for NCTC cells, with fetal calf serum instead of horse serum. Samples were the same as those previously described for the agar-diffusion test. Both Ti–6Al–4V discs and their extracts were evaluated. For the assay of extracts, 2×10^4 cells/well were exposed to the extracts of test specimens for 24 h. For the direct assay, test specimens were placed on the plate wells and a cell suspension of 3×10^4 cells/well was added. The exposure period was 48 h.

For both tests, once the exposure was terminated a qualitative and quantitative assessment of the cell cultures was performed. The cells were either washed with PBS and a morphological study was performed by optical microscopy, or they were trypsinized, stained with 7-amino-actinomycin D and quantified by flow cytometry.

2.3. Genotoxicity tests

2.3.1. Bacterial mutation assay

The mutagenic potential of the experimental Ti–6Al–4V material was examined with the reverse mutation test in *Escherichia coli* performed with the plate-incorporation method as described in Real Decreto 363/1995 [32]. *E. coli* WP2 *uvr* (pkM101) strain was provided by the Spanish Collection of Culture Types. Samples were extracted according to ISO 10993-12:2007 (Sample Preparation and Reference Materials) [33] as follows: the product (3 cm^2 sample/mL serum) was incubated with physiological serum under shaking at 37 °C for 72 ± 2 h. It was then sterilized by filtration and the resulting extract was used in the assay. Five different dilutions of the sample extract were used: 100%, 50%, 25%, 12.5% and 6.25%/plate. The vehicle was used as a negative control.

Briefly, 0.1 mL fresh bacterial culture and 0.1 mL physiological serum or the different concentrations of the sample extract were added to tubes with 2 mL top agar. To determine the effect of enzymatic activation, 0.5 mL S9 metabolic activation mixture was added to tubes similarly prepared. Then, tubes were gently vortexed and poured onto plates with ET_5 medium (E_5 medium supplemented with tryptophan). The bacterial survival test was performed in a similar way but using plates with Levine Eosine-Methylen Blue agar medium. Finally, plates were incubated at 37 ± 2 °C and examined after 48 h. Tryptophan-independent revertant colonies and viable cells were scored, respectively. The experiments were repeated at least twice and each concentration was tested in triplicate.

2.3.2. Cell-mutation assay

For this experiment, the established cell line mouse lymphoma L5178Y TK ± (ATCC CRL 9518) growing in suspension was used. The test sample underwent an extraction process as previously described (ISO 10993-12:2007). Six different dilutions of the sample extract were used: 100%, 50%, 25%, 12.5%, 6.25% and 3.125%/plate. Cell-culture medium was used as negative control. As positive controls, ethyl methanesulfonate (EMS, 0.25 and 0.5 μg/mL) and 7,12-dimethylbenzanthracene (7,12-DMBA, 5 and 7.5 μg/mL) were used with and without metabolic activation, respectively.

The test was performed according to OECD 476 guideline (1997) [34]. Cells deficient in thymidine kinase (TK) due to the mutation $TK^{+/-} \rightarrow TK^{-/-}$ are resistant to the cytotoxic effects of the pyrimidine analogue trifluorothymidine (TFT). Thymidine kinase-proficient cells are sensitive to TFT, which causes the inhibition of cellular metabolism and halts further cell division. Thus mutant cells are able to proliferate in the presence of TFT, whereas normal cells, which contain thymidine kinase, are not.

Cells in suspension were exposed to six sample concentrations, positive controls and negative control for 4 h both with and without metabolic activation. The treated cultures were maintained in growth medium for two days to allow near-optimal phenotypic expression of induced mutations. Mutant frequency was determined by seeding the cells in medium containing TFT to detect mutant cells, and in medium without TFT to determine the cloning efficiency (viability). After 10–14 days incubation time at 37 °C and 5% CO_2, colonies were counted. The mutant frequency was derived from the number of mutant colonies in selective medium and the number of colonies in non-selective medium.

2.4. Statistical analysis

Statistical analyses were performed by use of ANOVA and Student's *t*-tests. A $p < 0.05$ was considered significant. Experiments were repeated three times.

Table 1
Cytotoxicity test (ISO 7405) results obtained with the agar-overlay method. Negative control: HDPE (high-density polyethylene); positive control: PVC (polyvinyl chloride) with organic additives.

Sample	Cell response	Cytotoxicity
Control – (HDPE)	0/0	Not cytotoxic
Control + (PVC)	3/4	Mildly cytotoxic
Ti–6Al–4V discs	0/1	Not cytotoxic

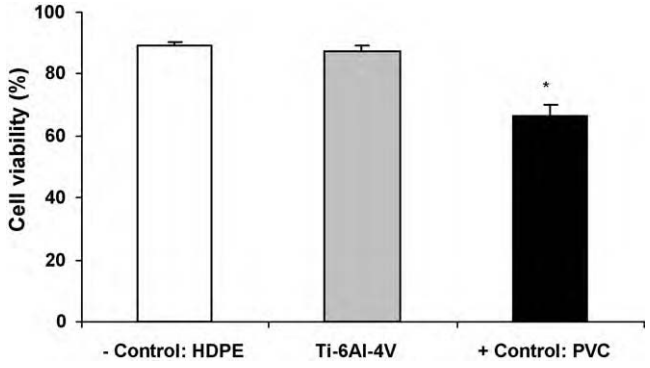

Fig. 2. Cytotoxicity test (ISO 7405) results obtained with the filter method. Cell viability (%) after 24-h exposure to extracts of the test specimen (Ti–6Al–4V discs), a negative control (high-density polyethylene, HDPE) and a positive control (polyvinyl chloride, PVC with organic additives). Experiments were performed three times and in duplicate per concentration. The significance level observed is $^*p < 0.05$ in comparison with the control group.

3. Results

3.1. Cytotoxicity tests

3.1.1. Cytotoxicity assessment according to guideline ISO 7405:2008

The agar-diffusion method showed that both the negative control and the test sample were not cytotoxic, whereas the positive control was mildly cytotoxic (Table 1). The filter-method resulted in 90% cell viability after exposure to Ti–6Al–4V discs, similarly to the results obtained with the negative control. The positive control, however, reduced the cell viability to 66% respect to the basal values (Fig. 2).

3.1.2. Cytotoxicity assessment according to guideline ISO 10993-5:2009

The morphology of MRC-5 cells exposed to extracts or directly to the test samples is shown in Fig. 3. Neither the test specimen nor the negative control induced any morphological alteration in the cells, whereas the cultures exposed to the positive control showed a reduction of cell number and cell death.

The qualitative evaluation showed no statistical difference in the cell number between the negative control and the test specimen in both methods. The positive control, however, significantly reduced the number of cells (Fig. 4).

3.2. Genotoxicity tests

3.2.1. Bacterial mutation assay

Ti–6Al–4V disc extracts did not induced mutagenic effects in *E. coli* at any of the concentrations assayed with or without metabolic activation. Moreover, no cytotoxicity was observed (Fig. 5).

3.2.2. Cell-mutation assay

The mutant frequency of the cells exposed to the different concentrations of the sample extracts with or without metabolic activation was never higher than 90 per 10^6 cells, i.e. the mutant

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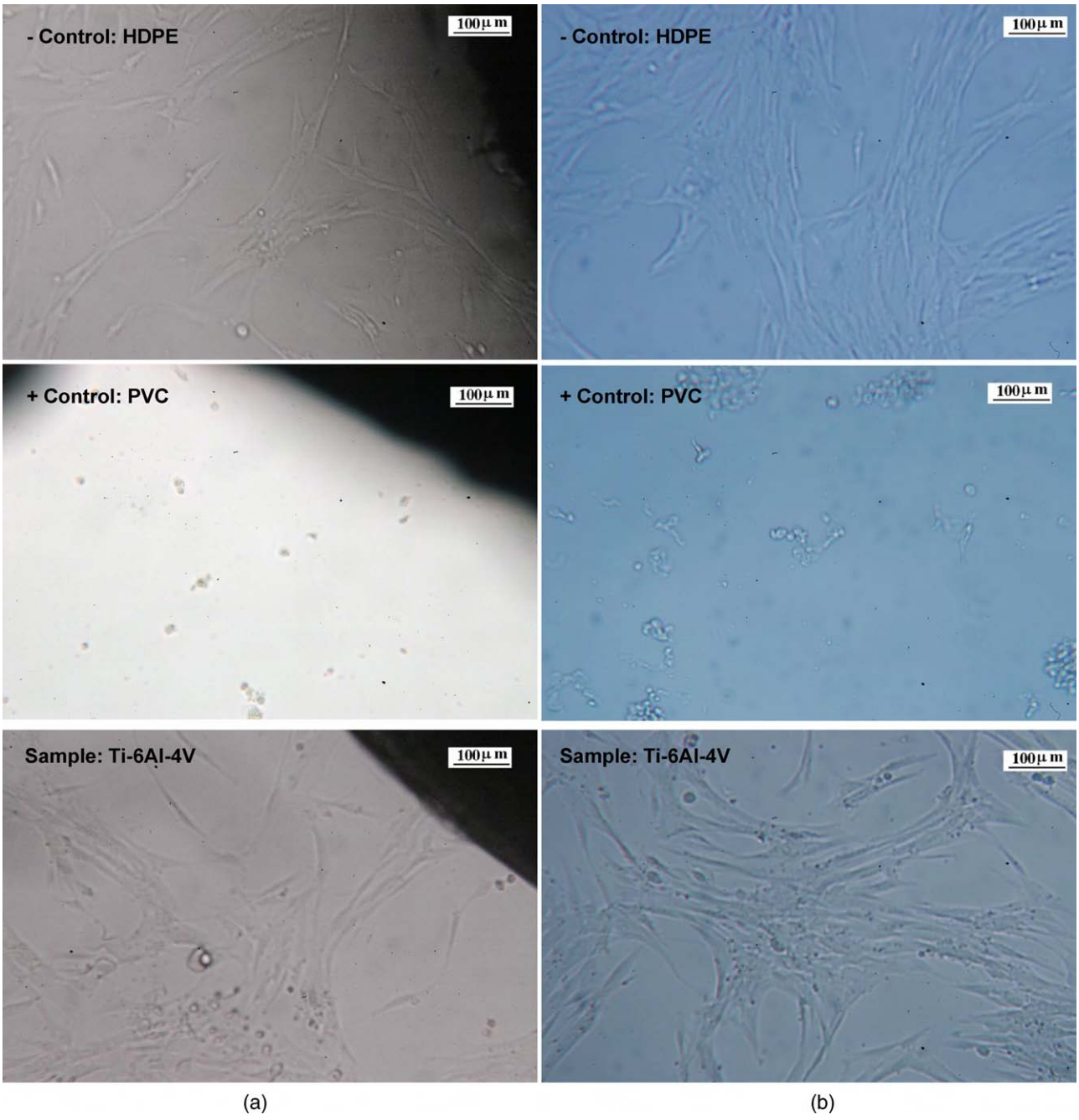

Fig. 3. Morphology of MRC-5 cells exposed to the test specimen (Ti–6Al–4V discs), a positive control (PVC with organic additives) and a negative control (HDPE): direct exposure (a) and exposure to their extracts (b). Cell cultures exposed to Ti–6Al–4V discs and their extracts do not show morphological differences in comparison with the negative control group. A reduction in cell number and cell death has been observed in the positive control group.

frequency of the negative control. Therefore Ti–6Al–4V discs are not mutagenic. Positive controls, however, showed mutagenic effects (Fig. 6).

4. Discussion

To date, titanium has been the most successful and the most often used dental implant material. The surface topography of dental implants plays a major role in the optimal interaction between the implant surface and the living tissue, generating a stable implant-tissue-connection. The successful application of medical implant devices requires modern test systems for evaluating acceptance by the host tissue, since these materials may release substances that could negatively influence the implant–host interaction (i.e. allergic reactions, non-integration). The intensity of such foreign-body reactions depends, among other factors, on the chemical and functional biocompatibility of the material. At the stage of preclinical evaluation of biocompatibility, different *in vitro* and *in vivo* test models are applied [35].

Titanium is a reactive metal. In air and aqueous electrolytes, it forms spontaneously a dense oxide film at its surface. This unwanted reaction product becomes a potent barrier against dissolution of the metal [36], and therefore Ti exhibits excellent resistance to corrosion. However, titanium dioxide is classified as possibly carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer [37].

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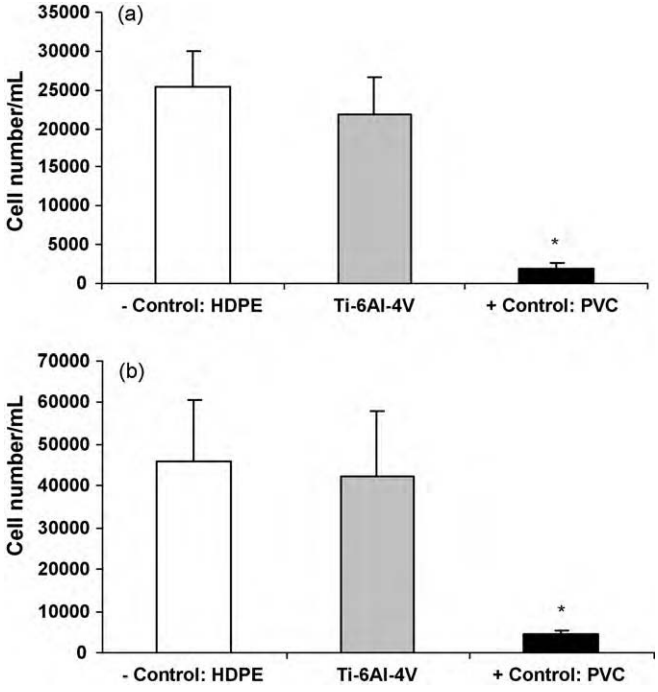

Fig. 4. Cytotoxicity test (ISO 10993-5) results obtained with the direct method (a) and the extraction method (b). Results are expressed as numbers of cells/mL. Test specimen: Ti–6Al–4V discs, positive control: PVC (polyvinyl chloride) with organic additives, negative control: HDPE (high-density polyethylene). Experiments were performed three times and in duplicate per concentration. The significance level observed is $^{*}p < 0.05$ in comparison with the control group.

As previously mentioned, a critical step for the clinical success of an implant is the process known as osseointegration, the direct apposition of bone tissue to the implanted material. This process is facilitated in biocompatible implants, which prevent the tissue in contact with the material from suffering the action of any element

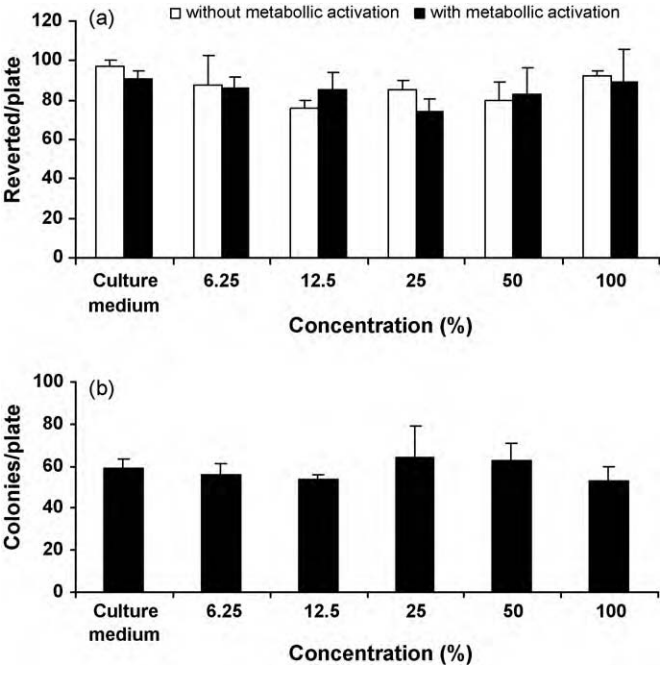

Fig. 5. Bacterial mutation assay of extracts of Ti–6Al–4V discs in *E. coli* with and without metabolic activation: (a) mutagenicity; (b) cytotoxicity. Experiments were performed three times and in duplicate per concentration.

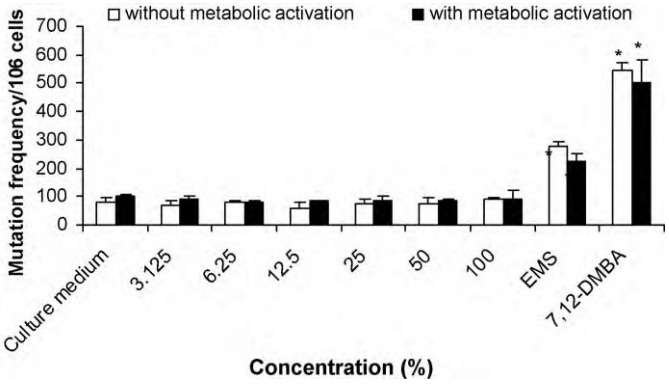

Fig. 6. Cell-mutation test of extracts of Ti–6Al–4V discs in L5178Y $Tk \pm$ cells with and without metabolic activation. Negative control: cell-culture medium, positive control 1: ethyl methanesulfonate (0.25 and 0.5 μg/mL), positive control 2: 7,12-dimethylbenzanthracene (5 and 7.5 μg/mL). Experiments were performed three times and in duplicate per concentration. The significance level observed is $^{*}p < 0.05$ in comparison with the control group.

that promotes an adverse cellular response such as inflammation, genotoxicity, or carcinogenicity [38,26]. Thus, the study of cytotoxic and genotoxic effects of dental materials is of great interest.

In the present investigation, the Ti–6Al–4V alloy has shown not to be cytotoxic neither in NCTC clone 929 mouse fibroblast (ISO 7405:2008) nor in MRC-5 human fibroblasts (ISO 10993-5:2009). Previously, Faria et al. [4] studied the cytotoxic effects of discs obtained from wax patterns (13-mm diameter and 4-mm thickness) that were cast from different alloys and commercially pure (cp) Ti on human oral squamous carcinoma SCC 9 cells, and found that cell viability was not affected by cpTi and Ti–6Al–4V. In contrast, Citeau et al. [16] examined the viability of osteoblasts in contact with titanium-alloy (Ti–6Al–4V) discs with different surfaces on MC3T3-E1 cells after 4, 8 and 15 days in culture, and observed that cell viability was decreased in contact with titanium discs at day 4, whatever the surface treatment. This reduction was almost completely restored as early as 8 days and totally abolished after 15 days in culture. The authors attributed this transient alteration of cell viability to the chemical composition. Aluminium-containing titanium alloys such as Ti–6Al–4V, exhibit an oxidized layer on their surfaces containing aluminium oxides that may exert some cytotoxic effects [39]. Also, Okazaki et al. [40] examined the biocompatibility of commercial pure Ti, Ti–6Al–4V and several new Ti alloys using MC3T3-E1 cells and observed that the growth ratio of these cells around Ti–6Al–4V was lower than that of cpTi because of the toxic effect of released vanadium ions. In this regard, Xavier et al. [41] reported that the nitric-acid treatment of the titanium surface (passivation) increased the release of chemical elements from the titanium surface. Sjögren et al. [23] evaluated the cytotoxicity of unalloyed titanium cast as discs (diameter 6 mm, height 2 mm, V-delta height 1 mm) sand-blasted with 110-μm alumina oxide using the agar-overlay method, the filter method and the MTT assay and in any of them observed toxicity. Other kinds of titanium alloy, such as nickel–titanium, have not shown cytotoxic, allergic or genotoxic activity [42,43]. Also, Ni–Cr alloy was evaluated and Wang and Li [25] found that it was biocompatible with mammalian cells.

However, direct and indirect induction of apoptosis in human mesenchymal stem cells (hMSCs) in response to titanium particles has been reported by Wang et al. [44]. The direct exposure to cpTi particles compromised cell viability through the induction of apoptosis, eliciting increased levels of the tumour suppressor proteins p53 and p73, in a manner dependent on material composition, particle dosage, and time. Additionally, conditioned medium collected from hMSCs exposed to cpTi particles was cytotoxic to hMSCs, inducing apoptosis in the absence of particles.

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The genotoxic potential of metallic titanium-based alloys has been studied in only a few investigations. Our results show that the Ti–6Al–4V material with the roughness characteristics previously described were not genotoxic using bacterial and cell-mutation assays. This finding agrees with studies performed by Wang and Li [25] who evaluated the mutagenicity and cytotoxicity of two experimental titanium alloys (Ti–Co and Ti–Ag) and cpTi by means of the Ames *Salmonella*/microsome mutagenicity spot test, the agar-diffusion method, and the cell-attachment assay. None of the samples were mutagenic. Also, Weber et al. [42] studied the cytotoxic, allergic and genotoxic activity of a nickel-titanium alloy and found a good short-term biological safety for this material. There are authors, however, who reported genotoxic effects of cpTi. Medeiros et al. [26] evaluated the genotoxic potential of a new titanium surface developed by plasma treatment using argon-ion bombardment, and compared it with an untreated titanium surface. Accordingly, the comet assay, the analysis of chromosomal aberrations (CAs), and the cytokinesis-block micronucleus (CBMN) assay were carried out with CHO-K1 cells grown on both titanium surfaces. The results show that the untreated titanium surface caused a significant increase in % tail moment, in the number of cells with CAs, tetraploidy, micronucleus frequency, and other nuclear alterations when compared with the negative control and with the plasma-treated titanium surface. Authors attributed this difference to increased surface roughness and changes in the thickness of the titanium-oxide layer. Therefore, the surface properties may influence the genotoxicity of the material, as observed in other cellular responses [26]. The aluminium-oxide blasting and nitric-acid passivation treatment on the studied material resulted in material characteristics that do not induce genotoxic responses. These results may be attributed to a thicker oxide layer, promoting surface hydration, which maintains the proteins adsorbed in this layer in their conformational state. In contrast, Medeiros et al. [26] observed genotoxicity from an untreated titanium surface due to lower hydrophilicity, allowing indirect interaction between the adsorbed proteins and the titanium surface ions, causing conformational changes in proteins that may have triggered events that promote genomic instability in the cell. Moreover, the hydrophilicity is affected not only by the chemical characteristics of the surface, but also by other topographical parameters, such as roughness and microtexture [45]. Zhu et al. [45] observed that osteoblastic cells grown on modified titanium surfaces with a thicker oxide layer and greater roughness adhered and proliferated better. Also, this thicker titanium-oxide layer provides a better resistance to corrosion, avoiding the occurrence of reduction/oxidation reactions. According to Medeiros et al. [26] the untreated titanium surface has a thinner oxide layer, and is consequently more susceptible to corrosion. The electrochemical process that occurs during corrosion of an untreated titanium surface would cause the release of titanium particles, ions, and unstable ions, which favour the emergence of free radicals involved in increased oxidative stress inside the cells, or DNA oxidation culminating in genotoxic effects.

In *in vivo* experiments, Piozzi et al. [46] observed the absence of cytotoxicity and genotoxicity of titanium. Rats underwent surgical implantation of titanium-alloy mini-plates in their tibias. Thirty, 90, and 180 days after implantation, the lung, liver, and kidney were removed for histopathological and genotoxic analysis as shown by hematoxylin–eosin stain and single-cell gel electrophoresis (comet) assay, respectively. No statistically significant differences in DNA damaging were found in all experimental groups when compared with the negative control for all organs evaluated. In addition, no remarkable morphological alterations were detected under histopathological analysis.

All these reports suggest that the toxic effects induced by cpTi and titanium alloys are dependent on several factors including composition, surface and size of the particle [26].

From the results obtained in this study it can be concluded that the commercial titanium-alloy Ti–6Al–4V material after aluminium-oxide sand-blasting and nitric-acid passivation, has a high biocompatibility with no cytotoxic effects on mouse and human fibroblasts, and it does not induce genotoxic responses in bacterial and cell-mutation assays.

Conflict of interest

No conflict of interest.

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