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

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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

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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.

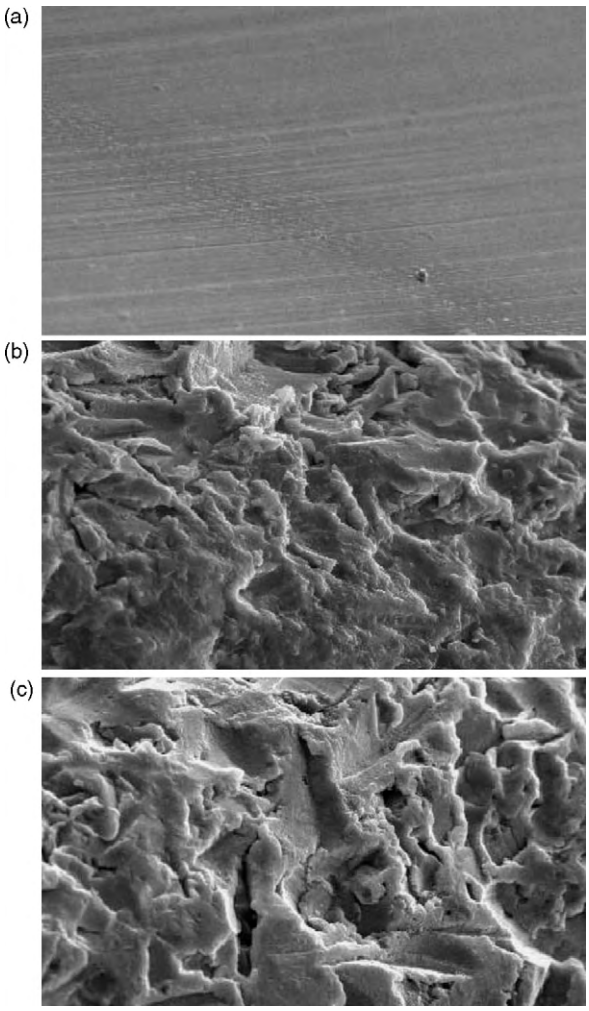

Fig. 1. SEM micrographs showing the surface roughness of the Ti–6Al–4V material: (a) as machined discs; (b) discs sand-blasted with aluminium oxide; and (C) sand-blasted discs after passivation with nitric oxide. Original magnification: 500×, 1000× and 1000×, respectively.

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 microscrope, 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].