



Assessment of the genetic risks of a metallic alloy used in medical implants

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Abstract

The use of artificial implants provides a palliative or permanent solution for individuals who have lost some bodily function through disease, an accident or natural wear. This functional loss can be compensated for by the use of medical devices produced from special biomaterials. Titanium alloy (Ti-6Al-4V) is a well-established primary metallic biomaterial for orthopedic implants, but the toxicity of the chemical components of this alloy has become an issue of concern. In this work, we used the MTT assay and micronucleus assay to examine the cytotoxicity and genotoxicity, respectively, of an extract obtained from this alloy. The MTT assay indicated that the mitochondrial activity and cell viability of CHO-K1 cells were unaffected by exposure to the extract. However, the micronucleus assay revealed DNA damage and an increase in micronucleus frequency at all of the concentrations tested. These results show that ions released from Ti-6Al-4V alloy can cause DNA and nuclear damage and reinforce the importance of assessing the safety of metallic medical devices constructed from biomaterials.

Key words: biomaterial, cytotoxicity, micronucleus, mutagenicity, Ti-6Al-4V.

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Introduction

Living organisms depend on inorganic elements for many vital processes. In humans, metal ions play a crucial role in homeostasis and small deviations from normal levels are indicative of system malfunctions or diseases (Anastassopoulou, 2003). Human exposure to metal derivatives results in acute to chronic toxicity that can lead to a wide range of diseases, including cancer (Guillamet *et al.*, 2008). The damage induced by metals includes the oxidative modification of ligands through metal-ligand coordination, as well as oxidative damage to neighboring molecules that are attacked by reactive intermediates produced in the initial reaction. Indeed, the chemical interaction between metallic compounds and biological molecules such as DNA can generate highly toxic products such as reactive oxygen species (ROS) (Valko *et al.*, 2006).

The use of artificial implants may be a palliative or permanent solution for individuals who have lost some bodily

function through disease, accidents or natural wear. This dysfunction can be compensated for by medical devices produced with special biomaterials. Stainless steel, cobalt-based alloys and titanium materials are widely used as implant materials in clinical practice, with each of these having specific advantages (Okazaki and Gotoh, 2005). However, the potential noxious effects of chemical elements present in the composition of medical implants, as well as debris released from these devices, are a constant concern in patients fitted with medical devices (Papageorgiou *et al.*, 2007). In particular, corrosion leading to the deterioration of metallic implants can release metal ion/corrosion products that are not biocompatible with the human body (Cadosch *et al.*, 2009). For this reason, there is an urgent need for studies dealing with metallic implants and the metal ions released from these structures into biological fluids (Sargeant and Goswami, 2007). The release or accumulation of toxic concentrations of metallic ions can result in cytotoxicity, inflammatory reactions and genotoxicity.

Titanium alloy (Ti-6Al-4V) is a well-recognized primary metallic biomaterial for orthopedic implants (Zreiqat *et al.*, 2005). Compared to other metals, this alloy shows

good biocompatibility, low toxicity, great stability with low corrosion rates and favorable mechanical properties. However, the toxicity of titanium alloy components, especially vanadium, has become an issue of concern (Okazaki and Gotoh, 2005). The release of aluminum and particularly vanadium ions from this alloy can generate long-term health problems such as peripheral neuropathy, osteomalacia and Alzheimers disease (Lin *et al.*, 2005).

Metals from orthopedic implants are released into surrounding tissue by various mechanisms, including corrosion, wear, and mechanically accelerated electrochemical processes such as stress corrosion, corrosion fatigue and fretting corrosion (Okazaki and Gotoh, 2005). In view of the significant risks associated with the use of metallic biomaterials in biological tissues, it is important to evaluate the potential of toxicity inherent in a metallic alloy. In this work, we examined the cytotoxicity and genotoxicity of an extract from Ti-6Al-4V using the MTT test and the micronucleus assay, respectively.

Materials and Methods

Preparation of Ti-6Al-4V extract

Rectangular pieces of Ti-6Al-4V (surface area: 18.7 cm²) were used to prepare the extract. Initially, the pieces were etched with a mixture of distilled water, hydrofluoric acid and nitric acid (5:1:3), according to ASTM E407, and then immersed in ethanol (15 min) and washed abundantly with distilled water. Each piece was then placed individually in a covered borosilicate flask and sterilized at 121 °C (15 min).

The extract was obtained by using standardized procedures and recommendations (ISO 10993-12). Hams F12 culture medium (Gibco) without fetal bovine serum (FBS) was added to borosilicate flasks containing the sterile pieces of Ti-6Al-4V alloy (1 mL of culture medium for each 1.2 cm² of metallic surface area) followed by incubation at 37 °C with orbital shaking (91 rpm). After six days (144 h), the culture medium (extract) was removed from the flasks and stored at -20 °C until tested. Pure culture medium used as a negative control was processed in a similar manner but without exposure to the metal alloy.

Cell culture

Chinese hamster ovary cells (CHO-K1) obtained from the Instituto Adolfo Lutz (São Paulo, Brazil) were used in this study. The cells were grown in 25 cm² flasks (TPP) containing 5 mL of Ham's F12 medium supplemented with 10% FBS, 1.2 g/L sodium bicarbonate (Sigma), antibiotics (penicillin 0.06 g/L; streptomycin 0.10 g/L; and kanamycin 0.05 g/L) at 37 °C in a humidified air atmosphere containing 5% CO₂.

Cell viability assay

Cell viability after exposure to the Ti-6Al-4V alloy extract was assessed by the methylthiazol tetrazolium

(MTT) assay. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble salt that can penetrate cells and mitochondria. The mitochondrial succinic dehydrogenase of viable cells subsequently converts this compound into insoluble formazan.

CHO-K1 cells were seeded (2.5 x 10⁴ cells/well) in two 96-well plates containing Ham's F-12 medium supplemented with 10% FBS. After 24 h to allow for cell fixation to the substrate, the plates were washed twice with phosphate-buffered saline (PBS) prior to use. Negative control wells received 100 µL of control medium whereas treated cells were incubated with 25%, 50%, 75% and 100% (v/v) of the extract (final volume: 100 µL/well). Two hours later, the plates were washed twice and 200 µL of complete culture medium was added to each well followed by incubation for 24 h and 48 h (one plate each). A 2 h incubation was also used for the micronucleus assay (see below) to allow direct correlation between the data from the two assays.

At the end of each incubation the medium was removed from the wells and discarded and the cells were washed twice with PBS. Each well then received 100 µL of MTT solution (0.5 mg/mL in PBS), including six wells without cells (blanks). The plates were incubated at 37 °C for 1 h followed by the addition of 200 µL of dimethylsulfoxide (DMSO) to each well and incubation with shaking at 37 °C for 20 min to ensure complete dissolution of the formazan crystals. The resulting absorbances were read at 570 nm in a microplate reader (Spectracount, Packard, USA) and cell viability was calculated using the equation:

$$\text{Cell viability (\%)} = \frac{A - B}{C - B} \times 100$$

where A is the absorbance of the treated group, B is the absorbance of the blank and C is the absorbance of the control group.

Micronucleus assay

The genotoxicity of the Ti-6Al-4V alloy extract was assayed using the micronucleus assay described by Wang and Huang (1994), with adaptations. CHO-K1 cells between the third and eighth passage were seeded in 35 mm culture dishes (2 x 10⁵ cells/dish) containing 1 mL of complete culture medium and a 20 x 20 mm coverslip. After 24 h (two complete cell cycles), the cells were washed twice with PBS and then incubated with 1 mL of control medium (negative control), 1 mL of mitomycin-C (0.3 µg/mL; positive control), a potent mutagenic agent, or 1 mL of Ti-6Al-4V extract at concentrations of 50%, 75% and 100% (v/v) for 2 h. The experiments were done three times, always without FBS.

After a 2 h incubation, the cells were washed twice with PBS and 1 mL of fresh complete medium containing cytochalasin-B (2.1 µg/mL) was added to the dishes. Cytochalasin-B prevented polymerization of the actin filaments

and blocked cytokinesis, resulting in the formation of binucleated cells. This binucleation corresponds to the occurrence of only one cell division. After 15 h, the cells were washed twice with PBS and treated with 1 mL of a hypotonic solution of 1% sodium citrate for 10 min followed by fixation in methanol:acetic acid (20:1, v/v) for 10 min prior to air drying. The coverslips containing adhered and fixed cells were stained with 5% Giemsa.

The frequency of micronucleation was determined in 1000 binucleated cells with a well-preserved cytoplasm. The number of cells containing 1-3 micronuclei out of the 1000 cells examined was scored using a Leica microscope (1000x magnification). The analyses were done by one of the co-authors (C.C.G.) who was blind to coverslip identification. The criteria used to identify micronuclei were those defined by Fenech (2000) and Titenko-Holland *et al.* (1997).

Frequency of binucleation

The influence of the Ti-6Al-4V extract on cell division was assessed by calculating the frequency of binucleation. The same slides prepared for the micronucleus assay were used and 500 cells with a well-preserved cytoplasm were counted. The frequency of binucleated cells was calculated as the proportion (percentage) of cells undergoing complete cell division after exposure to the extract.

Statistical analysis

The results were expressed as the mean \pm SD of the number of experiments indicated. Statistical comparisons of cell viability (MTT assay) and cell division indices were done using analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparisons test. The micronucleus assay results were analyzed by ANOVA followed by the Student-Newman-Keuls multiple comparisons test. Values of $p < 0.05$ indicated a significant difference. All statistical comparisons were done with InStat v. 3.00 (GraphPad Inc., La Jolla, CA, USA).

Results

Figure 1 shows the toxicity of the Ti-6Al-4V alloy extract in CHO-K1 cells based on the MTT assay. None of the concentrations tested had any significant effect on cell viability after incubation for 24 h and 48 h. In contrast, the micronucleus assay showed that all of the extract concentrations tested were genotoxic to these same cells and significantly increased the frequency of micronucleated cells (Table 1). However, there was no significant difference between cells treated with different concentrations of extract, *i.e.*, the genotoxicity was apparently not concentration-dependent.

Table 2 shows the effect of the Ti-6Al-4V alloy extract on cell division. Exposure to the extract reduced the

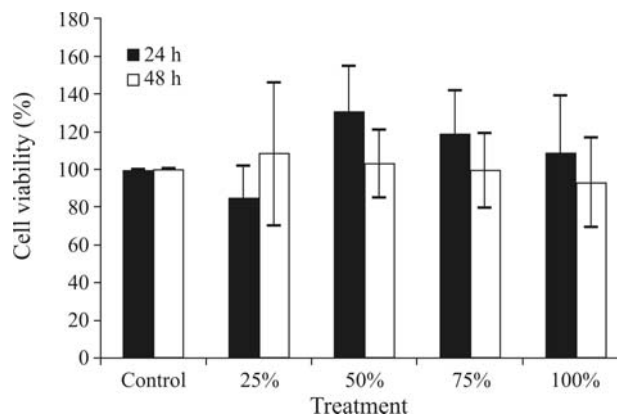


Figure 1 - Cytotoxicity of Ti-6Al-4V alloy extract in CHO-K1 cells. The cells were incubated with varying concentrations of extract for 24 h and 48 h, and cell viability was expressed as a percentage of control cell viability (cells incubated without extract). The columns are the mean \pm SD of three experiments.

mean frequency of binucleated cells, particularly at higher concentrations (75% and 100%).

Discussion

Ti-6Al-4V alloy is a metallic biomaterial widely used in medical and odontological implants, but there is little data in the literature on the biological risks associated with this alloy. Sedarat *et al.* (2001) used atomic absorption spectroscopy to investigate the release of metallic ions from this alloy over a period of 96 days in a solution that simulated the composition of body fluids. Aluminum and titanium release was constant throughout the 96 days whereas vanadium release occurred mainly during the first six days of the experiment.

More recently, Morais *et al.* (2009) reported the release of potentially important ions by Ti-6Al-4V *in vivo*. These workers studied the tissue accumulation of ions released from orthodontic mini-implants in rabbits after 1, 4 and 12 weeks and found low amounts of vanadium, tita-

Table 1 - Frequency of micronucleated cells (as an indicator of genotoxicity) in CHO-K1 cells incubated with three concentrations of Ti-6Al-4V alloy extract compared to control cells (incubated without extract).

Treatment	Experiment			Mean \pm SD
	I	II	III	
Control	11	17	15	14.3 \pm 3.06
Mitomycin-C	38	41	39	39.3 \pm 1.53*
Ti-6Al-4V extract (%)				
50	20	27	25	24.0 \pm 3.61*
75	17	24	25	22.0 \pm 4.36*
100	21	26	26	24.3 \pm 2.89*

The frequency of micronucleated cells was determined by analyzing 1000 binucleated cells per experiment (I, II and III). * $p < 0.01$ compared to control cells (not incubated with alloy).

Table 2 - Frequency (%) of binucleation in CHO-K1 cells incubated with three concentrations of Ti-6Al-4V alloy extract.

Treatment	Experiment			Mean \pm SD
	I	II	III	
Control	64	71	73	69.3 \pm 4.7
Mitomycin-C	39	44	47	43.3 \pm 4.0*
Ti-6Al-4V extract (%)				
50	55	61	63	59.7 \pm 4.2
75	48	54	57	53.0 \pm 4.6*
100	47	46	48	47.0 \pm 1.0*

The frequency (%) of binucleation was determined in 500 cells for each concentration of alloy extract. * $p < 0.01$ compared to the control cells incubated without extract.

nium and aluminum in the kidney, liver and lung. Okazaki *et al.* (2004) reported the release of ions from Ti-6Al-4V implants in rat tibia, with a significant increase in the Ti, Al and V concentrations in bone when compared with control rats. Vendittoli *et al.* (2010) reported the release of ions by different metallic devices (based on Cr-Co and TiNiVa alloys) used in hip joints in humans. The blood concentrations of various ions from these metallic devices were significantly greater than in individuals without devices, indicating a potential risk of intoxication by these ions. Ziaee *et al.* (2007) have shown that pregnant woman with metallic implants have higher levels of metal ions in their blood compared to pregnant women without implants, and that these ions can cross the placenta, thereby representing an important risk of intoxication for the fetus. However, despite the well-documented ability of the Ti-6Al-4V alloy to release ions, our results showed that the alloy extract was not cytotoxic, as judged by the MTT assay results in Figure 1. This finding agreed with that of Faria *et al.* (2008) who reported that Ti-6Al-4V alloy and pure commercial titanium were not cytotoxic *in vitro*.

In contrast, the Ti-6Al-4V extract induced DNA damage, as shown by micronucleus formation at all concentrations tested *in vitro* (Table 1). Micronucleus formation is a characteristic response to agents that cause DNA breakage (clastogenesis) or alter the physiology of the mitotic spindle, resulting in the loss of whole chromosomes during cell division (aneuploidy). Our results agree with those of Stea *et al.* (2000) and Doherty *et al.* (2001) who detected genetic alterations such as sister chromatid exchanges (SCE) and aneuploidies, respectively, in humans fitted with Ti-6Al-4V medical devices.

In view of the chemical composition of Ti-6Al-4V the risks associated with the use of this alloy *in vivo* should not be neglected. The release of potentially deleterious ions into biological fluids is a particularly important point to be highlighted. However, although many metals are genotoxic carcinogens in humans, the mechanisms involved in this genetic damage remain unclear (Guillamet *et al.*, 2008).

Based on studies with TiO₂, Lu *et al.* (1998) reported that titanium induces micronuclei and SCE in cultured CHO-K1 cells. In cultured Syrian hamster embryo cells (SHE line), TiO₂ also caused the formation of micronuclei and induced apoptosis, with the cytotoxicity being proportional to the concentrations tested (Rahman *et al.*, 2002). According to Reeves *et al.* (2008), TiO₂ nanoparticles produce ROS in biological systems and can cause genotoxic damage in cultured fish cells, probably through the production of hydroxyl radicals (\bullet OH). These authors also suggested that singlet oxygen reacted with the guanine moiety of cellular DNA to form 8-hydroxyguanine (8-OH-G), which is associated with \bullet OH that interacts non-selectively with all components of the DNA molecule to damage purine and pyrimidine bases and the deoxyribose backbone.

Long *et al.* (2007) studied the cytotoxicity of TiO₂ in cultured microglial cells (BV2) and neurons (N27). In BV2 cells, treatment with TiO₂ resulted in high ROS production, an increase in the expression of pro-apoptotic and pro-inflammatory genes, and a reduction in energy metabolism. In cultured N27 cells, energy metabolism was improved and pro-apoptotic caspases were activated, but no cytotoxicity was detected.

The controversy regarding the use of Ti-6Al-4V alloy in medical devices is related primarily to the recognized toxicity of vanadium, which can cause necrosis in cardiomyocytes, probably by altering the polarity of mitochondrial membranes (Soares *et al.*, 2008). The cytotoxicity and antiproliferative effects of different vanadium salts was demonstrated by Kordowiak *et al.* (2007) whereas Riley *et al.* (2003) showed that vanadium was the most toxic of several metals in pulmonary cells.

The European Food Safety Authority (EFSA, 2004) states that the intake of vanadium combinations can cause gastrointestinal disorders. In addition, other rat organs such as the kidneys are affected by relatively low doses of vanadium, whereas high doses of this metal affect reproduction and development in these animals. The genotoxicity of vanadium salts (SVO₅, Na₃VO₄ and NH₄VO₃) is seen in their ability to induce micronuclei *in vivo* (Cirani *et al.*, 1995). SVO₅, NH₄VO₃ and V₂O₃ also induce SCE and chromosomal aberrations in CHO cells (Owusu-Yaw *et al.*, 1990).

Ramirez *et al.* (1997) showed that V₂O₅ induces aneuploidy in human lymphocytes through its ability to interfere with the normal function of microtubules and fibers of the mitotic spindle. Other vanadium salts can also increase the numerical alterations and number of micronuclei in human cells (Migliore *et al.*, 1993). Together, these results suggest that a possible mechanism of action for the genotoxic effects of vanadium may be related to interference with the normal physiology of mitotic spindles. Léonard and Gerber (1994) proposed that vanadium is not clastogenic but affects mitosis and the distribution of chromosomes during cell division. However, vanadium can cause DNA breakage (clastogenesis) *in vivo*. In rats, V₂O₅

causes DNA breakage that can be detected with the comet assay in a variety of organs (liver, kidney, lung, spleen and heart), although there is considerable inter-organ variation in the extent of damage (Altamirano-Lozano *et al.*, 1999).

Aluminum is a well known toxic metal, especially in its trivalent form (Al^{3+}). Exposure to aluminum can result in pathogenic neurodegenerative disorders such as Parkinsons disease and Alzheimers disease (Lima *et al.*, 2007). These authors showed that $AlCl_3$ is genotoxic in human cells since all of the concentrations tested were cytotoxic and affected the mitotic index in different cell cycle phases. $AlCl_3$ can also cause DNA breakage and alterations in chromosomal number.

The blood cells of workers exposed occupationally to aluminum show a small increase in micronucleus formation when compared to control individuals (Crebelli *et al.*, 2002); these authors also showed that aluminum interferes negatively in DNA repair. The cytotoxicity and genotoxicity of aluminum were confirmed by Lankoff *et al.* (2006) who reported that this metal caused oxidative damage, cell cycle arrest and inhibition of DNA repair.

Positively charged metal ions can interact directly or indirectly with high electron density sites or negatively charged residues in DNA. Such sites in DNA are provided by the negatively charged phosphates of the backbone of both strands and the electron donors (nitrogen and oxygen) of DNA bases (Anastassopoulou, 2003). In the direct mechanism of action, the metal ion would coordinate completely with the DNA bases, while in the indirect mechanism, the ion would be at least partially coordinated by water molecules thereby allowing the formation of hydrogen bonds between these coordinated ligands and the negatively charged phosphates and other donor groups of DNA (Anastassopoulou, 2003). These suggestions would apply equally to ions derived from vanadium and titanium. Indeed, all of these metals have low-energy *d* orbitals that favor the formation of coordination bonds in metallic complexes.

As shown here, the incubation of CHO-K1 cells with Ti-6Al-4V alloy extract resulted in a significant reduction in the mitotic index, particularly at the higher concentrations tested. The decrease in the frequency of binucleation indicated that the extract interfered with cell division, probably through its ability to damage DNA. A variety of stress inducers, including DNA-damaging agents, activate checkpoint functions in cells, leading to cell cycle arrest. These checkpoints have surveillance mechanisms to detect specific DNA conformations indicative of damage or ongoing repair and replication (Chen and Shi, 2002). The cell cycle arrest caused by the Ti-6Al-4V alloy extract may explain why the extract was not cytotoxic to these cells. The DNA damage detected in the micronucleus assay has the potential to trigger apoptotic pathways, but at the time intervals analyzed in the MTT assay (24 h and 48 h) these pathways may not have been sufficiently activated to cause cell death.

Apart from their genotoxic effects, metals can directly influence the cell cycle. Vanadium, for instance, inhibits protein phosphatase (Scheving *et al.*, 1999). Inhibition of the phosphatase CDC25 family members by metals can delay the cell cycle transition (Chen and Shi, 2002) and, consequently reduce the mitotic index *in vitro*, as observed here.

Although the Ti-6Al-4V alloy extract was not cytotoxic to CHO-K1 cells, its components can potentially induce apoptosis, necrosis and interfere with cellular proliferation. Although the biochemical mechanisms responsible for this action are not well established, our findings suggest that damage to DNA is a central event in the final outcome. Since many metals or metal-containing compounds are involved in human pathologies such as cancer (Chen, 2002), the development of biomaterials for use in medical devices requires careful analysis of the potential toxicity of metal ions released by metallic alloys. The biological safety of metals and alloys is therefore a major factor in assessing the potential application of these materials.

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