Cytotoxicity of Titanium and Titanium Alloying Elements

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ABSTRACT

It is commonly accepted that titanium and the titanium alloying elements of tantalum, niobium, zirconium, molybdenum, tin, and silicon are biocompatible. However, our research in the development of new titanium alloys for biomedical applications indicated that some titanium alloys containing molybdenum, niobium, and silicon produced by powder metallurgy show a certain degree of cytotoxicity. We hypothesized that the cytotoxicity is linked to the ion release from the metals. To prove this hypothesis, we assessed the cytotoxicity of titanium and titanium alloying elements in both forms of powder and bulk, using osteoblast-like $SaOS_2$ cells. Results indicated that the metal powders of titanium, niobium, molybdenum, and silicon are cytotoxic, and the bulk metals of silicon and molybdenum also showed cytotoxicity. Meanwhile, we established that the safe ion concentrations (below which the ion concentration is non-toxic) are 8.5, 15.5, 172.0, and 37,000.0 μg/L for molybdenum, titanium, niobium, and silicon, respectively.

KEY WORDS: cytotoxicity, titanium, titanium alloy elements, metal powder.

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INTRODUCTION

Titanium and some of its alloys, such as Ti6Al4V and TiNi, have found extensive applications in dentistry and orthopedics (Williams, 1982; Li *et al*., 2000; Chu *et al*., 2005). The release of metal ions from titanium alloy implants may generate an adverse biological effect or elicit allergic reactions. Vanadium (V) and aluminum (Al) in the Ti6Al4V alloy were shown to be potentially cytotoxic, and, over time, the alloy produced adverse reactions in the body tissues (Okazaki *et al*., 1998a,b). Nickel has also been reported to be allergenic and possibly carcinogenic to humans (Köster *et al.,* 2000). The elastic modulus of dense titanium implants (80-130 GPa) (Long and Rack, 1998) is much higher than that of natural bone (0.1-20 GPa) (Wang *et al.,* 2007), which causes stress shielding, leading to the implant loosening, and contributes to the early failure of the implant material (Williams, 1982; Niinomi, 1998). Consequently, extensive research has been carried out to develop new titanium alloys with a lower elastic modulus. Molybdenum (Mo), niobium (Nb), tantalum (Ta), silicon (Si), zirconium (Zr), and tin (Sn) are commonly used to develop new Ti alloys (Wang, 1996; Antipov and Moiseev, 1997; Kuroda *et al.,* 1998; Niinomi, 1998; Kasuga *et al.,* 2003; Gogia, 2005).

Increased interest in the use of porous Ti and Ti alloy scaffolds for biomedical applications has also been observed in recent years, due to the potential of porous Ti and Ti alloy scaffolding to provide bone-mimicking properties, including a low elastic modulus close to that of bone, and new bone tissue ingrowth ability and vascularization (Gibson and Ashby, 1997; Wen *et al.,* 2001; Dunand, 2004). Powders of Ti and Ti alloying elements are used to fabricate porous Ti and Ti alloy scaffolds with controlled pore shape, pore size, and porosity through powder metallurgy (Wen *et al.,* 2002a,b; Oh *et al*., 2003a,b). Meanwhile, metals may exhibit different toxicity levels in their bulk and powdered forms. To date, research on the biological properties of Ti and Ti alloying metals has been focused on their bulk forms that were prepared by methods other than powder metallurgy (*e.g*., the ingot method) (Steinemann, 1980, 1999; Okazaki *et al.,* 1998a,b; Köster *et al.,* 2000; Okazaki and Nishimura, 2000; Okazaki and Gotoh, 2005), while little is known about the biological properties of metal powders and the effects of metal powders on cellular behavior (Pypen *et al*., 1998).

It is commonly accepted that titanium and the titanium alloying elements of tantalum, niobium, zirconium, molybdenum, tin, and silicon are biocompatible. However, our research in the development of new titanium alloys for biomedical applications indicated that some titanium alloys containing Mo, Nb, and Si, produced by powder metallurgy, show a certain degree of cytotoxicity. In this study, the cytotoxicity of Ti and the most commonly used titanium alloying elements, Ta, Nb, Zr, Sn, Mo, and Si, was assessed in both the bulk and powdered forms, with $SaOS_2$ cells. The cell morphology and adhesion to the Ti, Nb, Zr, Sn, Mo, and Si discs were observed by confocal microscopy. The cytotoxicity of the elemental metals was linked to the metal

ion concentrations, and safe concentration limits were determined.

MATERIALS & METHODS

Preparation of Specimens

The bulk elemental metals of Ti, Ta, Nb, Zr, Sn, Si, and Mo were prepared by arc-melting of the respective elemental metal powders (all with purity of 99.8% and particle size of 325 mesh; Atlantic Equipment Engineers, Bergenfield, NJ, USA) in an argon atmosphere. The ingots of the elemental metals were cut into discs of 14 mm in diameter and 2 mm in thickness. The surfaces of the discs were ground with silicon carbide papers with grits of 240 and 600 sequentially. The discs were then ultrasonically cleaned in the following sequence: distilled water, 100% acetone, distilled water, 100% ethanol, and distilled water for 10 min at each step. All bulk discs were sterilized in 70% ethanol for 2 hrs in an orbital shaker and air-dried at room temperature in a biohazard hood for 2 hrs.

Cell Culture

Osteoblast-like cells ($SaOS_2$) (Barwon Biomedical Research, Geelong Hospital, Victoria, Australia), a human osteosarcoma cell line with osteoblastic properties, were cultured in minimum essential media (MEM) (Gibco, Invitrogen, Mulgrave, VIC, Australia) supplemented with 10% fetal bovine serum (Bovogen Biologicals, Essendon, VIC, Australia), 1% non-essential amino acid (Sigma-Aldrich, Castle Hill, NSW, Australia), 10,000 units/mL penicillin-10,000 μg/mL streptomycin (Gibco), and 0.4% amphostat B (In Vitro Technologies, Auckland, New Zealand) at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium was changed every 3 days.

Cytotoxicity

The cytotoxicity of all elemental metal samples was evaluated by use of the media extracts of the samples (International Organization for Standardization, 1999). The sterilized bulk samples were immersed in the media at a ratio of 3.6 cm^2/mL (the surface area of disc to volume of media). Alloy element powders were mixed with the media at a concentration of 0.22 g/mL. All samples (discs and powders with media) were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 72 hrs. The discs were removed from the media, and the powdered mixture was filter-sterilized with a 0.22-μm filter (Falcon, BD Biosciences, San Jose, CA, USA) to obtain the extracts. Cells were also seeded into wells containing only the media and incubated for 72 hrs as a negative control.

$SaOS_2$ cells were seeded into negative controls and extracts from the discs and powders at a density of 10,000 cells *per* well. After cell culture for 5 days, cells were harvested with 0.1% Trypsin-5 mM EDTA (Sigma-Aldrich) and collected. Cell counts were obtained by the trypan blue exclusion method, whereby dead cells are stained blue and live cells remain clear. The cell viability was determined by the ratio of live cells to the total number of cells *per* sample (Kruse and Patterson, 1973).

The metallic ion concentrations in the extracts of Ti, Nb, Mo, and Si powders were measured by inductively coupled plasma atomic emission spectroscopy (Varian Vista AX Simultaneous CCD, Palo Alto, CA, USA) and inductively coupled plasma mass spectroscopy (Agilent 4500, Palo Alto, CA, USA), according to the ANSTO method ENV-I-035-027 and ENV-I-035-026 (Australian Nuclear Science and Technology Organisation, Lucas Heights, NSW, Australia).

Cell Morphological Analysis

After cell culture for 5 days, the cell-seeded discs were fixed with 2% paraformaldehyde and permeabilized with 0.2%(v/v) triton-X100 in phosphate-buffered saline (PBS) (Sigma-Aldrich) for 10 min each at room temperature. The discs were then incubated with 1% phalloidin and 4'-6-diamidino-2-phenylindole (DAPI) overnight at 4°C. Three washes with PBS were included between each of the steps mentioned. The cell morphology of $SaOS_2$ cultured on surfaces of bulk element metals was observed by confocal microscopy (Leica SP5, Leica Microsystems, Wetzlar, Germany).

Statistical Analysis

The values are expressed as means ± standard deviation (SD). All of the experiments were conducted in triplicate. We used one-way ANOVA (SPSS 14.0 for Windows software) to determine the differences observed among the groups; $p < 0.05$ was considered significant.

RESULTS

Cytotoxicity of Elemental Metal Powders

The metal powders of Ti, Ta, Nb, Zr, Sn, Si, and Mo showed different degrees of cytotoxicity after cell culture for 5 days (Fig. 1). The control group (cells seeded into wells containing only media) was considered biocompatible. The elemental metals could also be considered biocompatible if the cell viability on the metals was equivalent to or greater than that of the control group. The cell viability for the metal powders of Ta, Zr, and Sn was 78 ± 2%, 76 ± 3%, and 77 ± 6%, respectively, compared with that of the control group, which was 84 ± 5% ($p > 0.05$). It could be seen that the metal powders of Ta, Zr, and Sn showed biocompatibility compared with the control group. It was noticeable that the cell proliferations in these groups of powder samples were lower than that of the control group, displaying an increase of 0.5- to 0.7-fold above the initial density of 10,000 cells, whereas the control group showed an increase of 1.8-fold. The metal powder of Nb group displayed a moderate cell viability of 66 ± 3%, but the cell proliferation rate on the Nb powders was 0.9 ± 0.1, compared with that of the control group, 2.8 ± 0.4. It could be seen that the $SaOS_2$ cells were inhibited in the extract of Nb powder. The cell viabilities for the metal powders of Ti, Si, and Mo groups were 43 ± 4%, 2 ± 0.7%, and 11 ± 3%

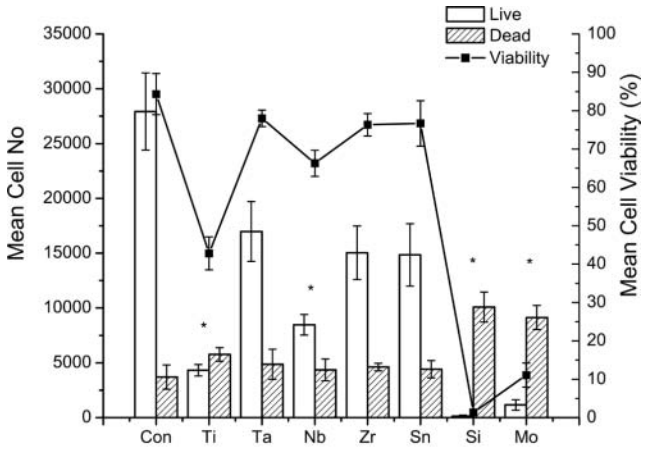

Figure 1. Mean ± SD cell number (left, Y axes) of live and dead cells, and mean ± SD cell viability (right, Y axes) in extracts of elemental metal powders of Ti, Ta, Nb, Zr, Sn, Si, and Mo after cell culture for 5 days. N = 3. *Significantly different from the control, $p < 0.05$.

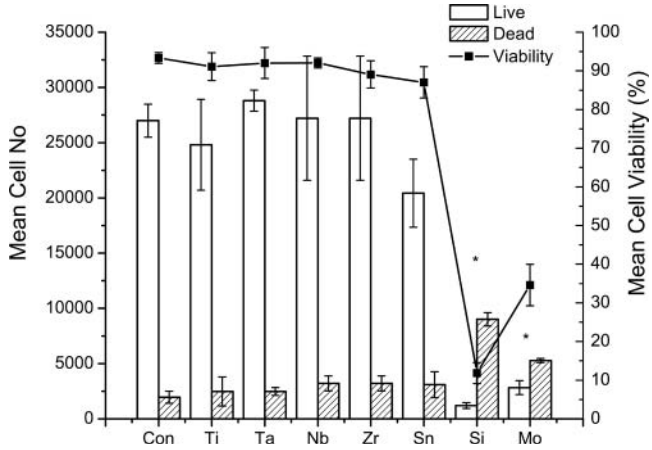

Figure 2. Mean ± SD cell number (left, Y axes) of live and dead cells, and mean ± SD cell viability (right, Y axes) in extracts of elemental bulk metals of Ti, Ta, Nb, Zr, Sn, Si, and Mo after cell culture for 5 days. N = 3. *Significantly different from the control, $p < 0.05$.

($p < 0.05$), respectively, and cell proliferation was not observed in this group of powder samples. It can be concluded that the metal powders of Ti, Si, and Mo were cytotoxic.

Cytotoxicity of Bulk Elemental Metals

The cell viability for the bulk metals of Ti, Ta, Nb, Zr, and Sn groups ranged from 90 ± 3% to 92 ± 4%, comparable with that of the control group, 93 ± 2% ($p > 0.05$), and the cell viability for the bulk metals of Mo and Si groups was 34 ± 5% and 12 ± 3%, respectively (Fig. 2). It could be seen that the bulk metals of Ti, Ta, Nb, Zr, and Sn exhibited excellent biocompatibility, while the bulk Si and Mo showed substantial cytotoxicity after cell culture for 5 days. It was also noted that the biocompatibility of the bulk metal of Sn was slightly inferior to that of the bulk metals of Ti, Ta, Nb, and Zr. The $SaOS_2$ cells cultured in the extracts of Ti, Ta, Nb, and Zr discs proliferated at similar rates, with an increase of 1.5- to 1.7-fold compared with the control group of 1.7-fold increases, while the cell proliferation rate in the Sn extract was slower, with an increase of 1.1-fold.

Morphology of Cells

The cell attachment and morphology on the elemental metals of Ti, Ta, Nb, Zr, Sn, Si, and Mo in their bulk forms after cell culture for 5 days were observed by confocal microscopy (Fig. 3). The $SaOS_2$ cells had flattened and spread on the surfaces of Ti, Ta, Nb, and Zr. Although the cells were able to attach to Sn, only a small number of cells were observed. In contrast, the cells attached to Si and Mo were small and shrunken, generally exhibiting the characteristics of unhealthy cells. It can be concluded that the bulk elemental metals of Ti, Ta, Nb, and Zr exhibited excellent biocompatibility, the bulk metal of Sn exhibited moderate biocompatibility, and the bulk metals of Si and Mo exhibited substantial cytotoxicity. The cell morphology observations are consistent with the cytotoxicity test results.

Safe Ion Concentration

The cytotoxicity assessment demonstrated that the metal powders of Ti, Nb, Mo, and Si were toxic, but the bulk Ti and Nb were biocompatible; meanwhile, the bulk Mo and Si were less cytotoxic compared with their powder counterparts. The cytotoxicity of the metal powders may be linked to the ion release from the metals, because the ion concentrations in the extracts of the bulk and powdered metals are measured significantly differently. Therefore, it is necessary to determine the safe ion concentrations of the elemental metals of Ti, Nb, Mo, and Si, below which the material is biocompatible for biomaterial applications. To obtain the safe ion concentrations for the metal powders of Ti, Nb, Mo, and Si, we assessed the extracts in 5 different ion concentrations: (i) undiluted, (ii) 1:2 dilution, (iii) 1:4 dilution, (iv) 1:8 dilution, and (v) 1:16 dilution. The cell viability and proliferation increased with the decrease of the metallic ion concentrations in the extracts (Fig. 4). It can be seen that the biocompatibility of the extracts with different metal powder concentrations was significantly improved by the decrease of the metal ion concentrations. The cell proliferation and viability reached a plateau when the metal ion concentration was lower than a certain concentration. When this threshold was reached, the cell proliferation and viability were comparable with those of the control group, a benchmark of good biocompatibility and non-toxicity. The threshold of metal ion concentrations was determined to be 15.5 μg/L for Ti, 8.5 μg/L for Mo, 172.0 μg/L for Nb, and 37,000.0 μg/L for Si (Fig. 4). It can be concluded that the biocompatibility of Ti alloys can be achieved when the metal ion concentrations are below these values.

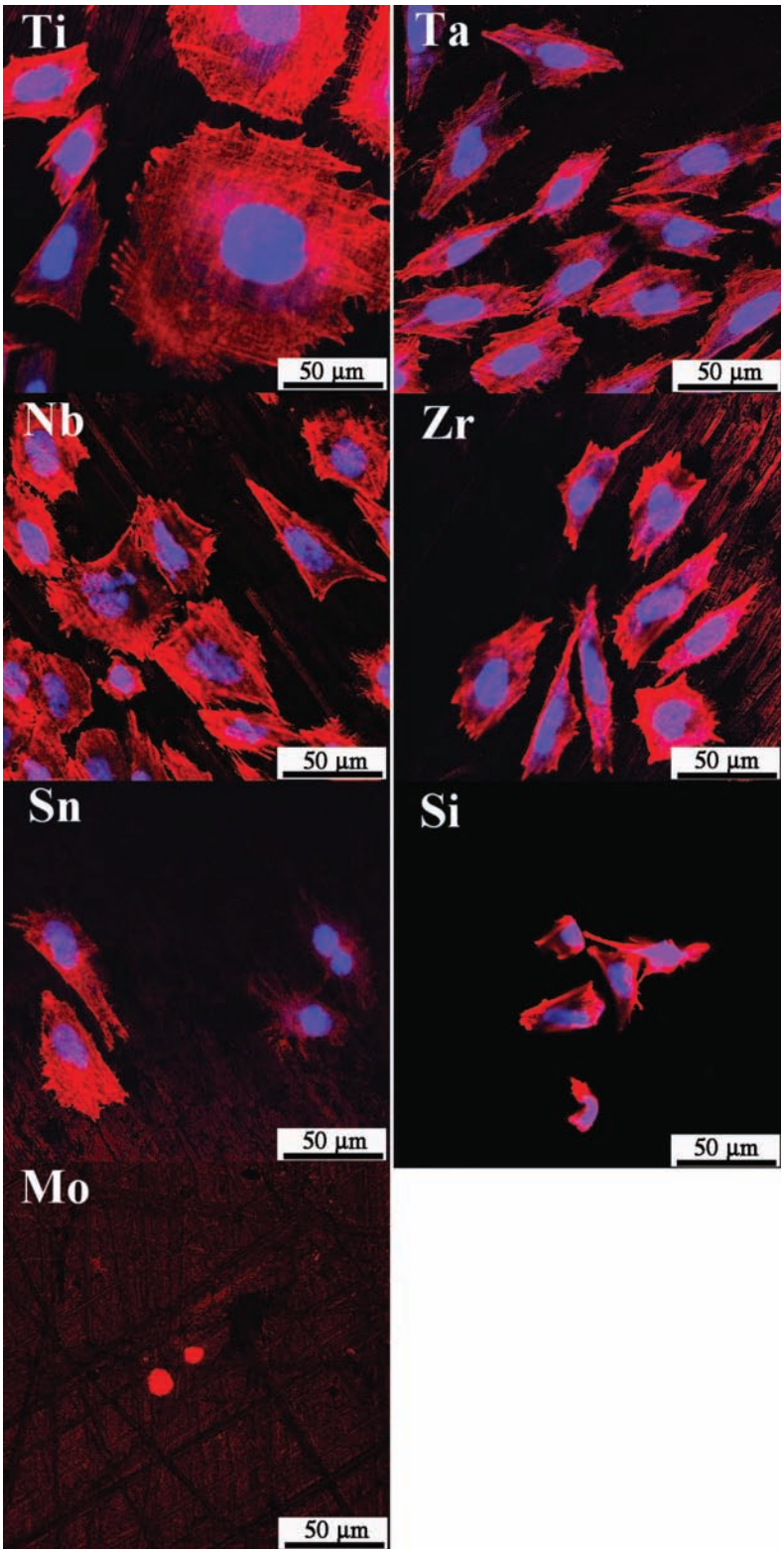

Figure 3. Confocal microscope images showed that $SaOS_2$ cells grew on elemental metals of Ti, Ta, Nb, Zr, Sn, Si, and Mo after cell culture for 5 days.

DISCUSSION

The release of metal ions from dental and orthopedic implants directly affects their biocompatibility. It has been reported that ion release from implants into the surrounding tissue may cause various problems such as osteolysis, cutaneous allergic reactions, remote site accumulation, and, eventually, the failure of the implants (Takeda *et al*., 1989; Wapner, 1991; Okazaki and Nishimura, 2000; Brunette *et al*., 2001; Geurtsen, 2002; Manaranche and Hornberger, 2005).

The present study demonstrated that the elemental metals exhibited different cytotoxicities in the forms of bulk and powder. The experimental results indicated that the elemental metal powders of Ti and Nb showed substantial cytotoxicity, but the bulk Ti and Nb showed biocompatibility. The elemental metal powders of Mo and Si exhibited relatively strong cytotoxicity, while the bulk Mo and Si showed substantial cytotoxicity. Meanwhile, the elemental metals of Ta, Zr, and Sn exhibited good biocompatibility in both forms of bulk and powder. However, a reduction in the degree of cytotoxicity was observed in the bulk metals when compared with their powdered forms. This is because the bulk elemental metals released a significantly lower ion concentration into the media compared with their powdered counterparts. This lower metal ion concentration produced lower cytotoxicity (*e.g.,* Mo and Si), or led to non-cytotoxicity completely (*e.g*., Ti and Nb).

We have found that there is a safe ion concentration for the elemental metals below which the extract of the metal powders is non-toxic. The safe ion concentrations of the elemental metal powders for Mo, Ti, Nb, and Si are 8.5, 15.5, 172.0, and 37,000.0 µg/L, respectively. The elemental metal of Mo exhibited the lowest safe ion concentration and showed substantial cytotoxicity even in the bulk form. These results indicated that the Mo contents should be limited to a certain level in the design and development of new Ti alloys for dental and orthopedic applications. In conclusion, these new findings provide fundamental knowledge and new insights for future design and development of new biocompatible titanium alloys for dental and orthopedic applications.

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REFERENCES

Antipov AI, Moiseev VN (1997). Coefficient of β-stabilization of titanium alloys. *Metal Sci Heat Treatment* 39:499-503.

Brunette DM, Tengvall P, Textor M, Thomsen P. editors (2001). Titanium in medicine. Berlin, Germany: Springer-Verlag.

Chu CL, Chung CY, Zhou J, Pu YP, Lin PH (2005). Fabrication and characteristics of bioactive sodium titanate/titania graded film on NiTi shape memory alloy. *J Biomed Mater Res A* 75:595-602.

Dunand DC (2004). Processing of titanium foams. *Advanced Eng Mater* 6:369-376.

Geurtsen W (2002). Biocompatibility of dental casting alloys. *Crit Rev Oral Biol Med* 13:71-84.

Gibson LG, Ashby MF (1997). Cellular solids: structure and properties. Cambridge, UK: Cambridge University Press.

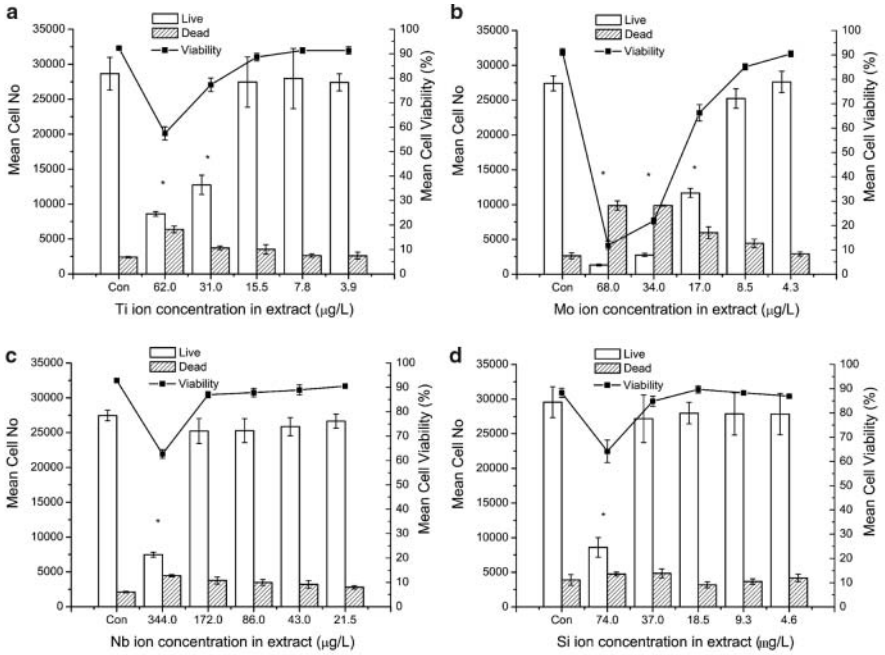

Figure 4. Mean ± SD cell number (left, Y axes) of live and dead cells, and mean ± SD cell viability (right, Y axes) in extracts of elemental metals of Ti, Ta, Nb, Zr, Sn, Si, and Mo with different metal ion concentrations after cell culture for 5 days. N = 3. *Significantly different from the control, $p < 0.05$.

Gogia AK (2005). High temperature titanium alloys. *Defence Sci J* 55: 143-173.

International Organization for Standardization (1999). Biological evaluation of medical devices—Part 5: Test for *in vitro* cytotoxicity. Arlington, VA: ANSI/AAMI ISO 10993-5.

Kasuga T, Nogami M, Niinomi M, Hattori T (2003). Bioactive calcium phosphate invert glass-ceramic coating on b-type Ti-29Nb-13Ta-4.6Zr alloy. *Biomaterials* 24:283-290.

Köster R, Vieluf D, Kiehn M, Sommerauer M, Kähler J, Baldus S, *et al*. (2000). Nickel and molybdenum contact allergies in patients with coronary in-stent restenosis. *Lancet* 356:1895-1897.

Kruse PF, Patterson MK (1973). Tissue culture, methods and application. New York, USA: Academic Press.

Kuroda D, Niinomi M, Morinaga M, Kato Y, Yashiro T (1998). Design and mechanical properties of new β type titanium alloys for implant materials. *Mater Sci Eng A* 243: 244-249.

Li BY, Rong LJ, Li YY, Gjunter VE (2000). Synthesis of porous Ni-Ti shape-memory alloys by self-propagating high-temperature synthesis: reaction mechanism and anisotropy in pore structure. *Acta Mater* 48: 3895-3904.

Long M, Rack HJ (1998). Titanium alloys in total joint replacement—a materials science perspective. *Biomaterials* 19:1621-1639.

Manaranche C, Hornberger H (2005). Corrosion and biocompatibility of dental alloys. *Eur Cell Mater* 9(Suppl 1):35-36.

Niinomi M (1998). Mechanical properties of biomedical titanium alloys. *Mater Sci Eng A* 243:231-236.

Oh IH, Nomura N, Masahashi N, Hanada S (2003a). Mechanical properties of porous titanium compacts prepared by powder sintering. *Scripta Mater* 49:1197-1202.

Oh IH, Nomura N, Masahashi N, Hanada S (2003b). Microstructures and mechanical properties of porosity-graded pure titanium compacts. *Mater Trans* 44:657-660.

Okazaki Y, Gotoh E (2005). Comparison of metal release from various metallic biomaterials *in vitro*. *Biomaterials* 26:11-21.

Okazaki Y, Nishimura E (2000). Effect of metal released from Ti alloy wear powder on cell viability. *Mater Trans JIM* 41: 1247-1255.

Okazaki Y, Rao S, Asao S, Tateishi T, Katsuda S, Furuki Y (1998a). Effects of Ti, Al and V concentrations on cell viability. *Mater Trans JIM* 39:1053-1062.

Okazaki Y, Rao S, Tateishi T, Ito Y (1998b). Cytocompatibility of various metal and development of new titanium alloys for medical implants. *Mater Sci Eng A* 243:250-256.

Pypen CM, Dessein K, Helsen JA (1998). Comparison of the cytotoxicity of molybdenum as powder and as alloying element in a niobium-molybdenum alloy. *J Mater Sci Mater Med* 9:761-765.

Steinemann SG (1980). Corrosion of surgical implants—*in vivo* and *in vitro* tests. In: Evaluation of biomaterials, advances in biomaterials. Winter GD, Leray JL, de Groot K, editors. Chichester: Wiley, pp. 1-34.

Steinemann SG (1999). Compatibility of titanium in soft and hard tissue—the ultimate is osseointegration. In: Materials for medical engineering, Euromat '99. Stallforth H, Revell P, editors. Weinheim, Germany: Wiley-VCH, pp. 199-203.

Takeda S, Kakiuchi H, Doi H, Nakamura M (1989). Cytotoxicity of pure metals. *Jpn Soc Dent Mater Devices (JSDMD)* 8:648-652 [in Japanese].

Wang K (1996). The use of titanium for medical applications in the USA. *Mater Sci Eng A* 213:134-137.

Wang XJ, Li YC, Hodgson PD, Wen CE (2007). Nano- and macro-scale characterisation of the mechanical properties of bovine bone. *Mater Forum* 31:156-159.

Wapner KL (1991). Implications of metallic corrosion in total knee arthroplasty. *Clin Orthop Relat Res* 271:12-20.

Wen CE, Mabuchi M, Yamada Y, Shimojima K, Chino Y, Asahina T (2001). Processing of biocompatible porous Ti and Mg. *Scripta Mater* 45:1147-1153.

Wen CE, Yamada Y, Shimojima K, Chino Y, Asahina T, Mabuchi M (2002a). Processing and mechanical properties of autogenous titanium implant materials. *J Mater Sci Mater Med* 13:397-401.

Wen CE, Yamada Y, Shimojima K, Chino Y, Hosokawa H, Mabuchi M (2002b). Novel titanium foam for bone tissue engineering. *J Mater Res* 17:2633-2639.

Williams DF (1982). Titanium and titanium alloys: biocompatibility of clinical implant materials. Vol. 1. Boca Raton, FL: CRC Press Inc.