Chula Med J Vol. 63 No. 2 April - June 2019; 89 - 93

DOI : 10.14456/clmj.1476.13

Original article

In vitro biocompatibility of novel titanium-based amorphous alloy thin film in human osteoblast-like cells

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Background: Toxic free Ti-based amorphous alloy has the potential to be used in biomedical fields due to its excellent biocompatibility and osseointegration.
Objectives: The purpose of this study was to develop a series of Ti44Zr10Pd10Cu6+ ×Co23-×Ta7 (× = 0, 4, 8) and examine their biocompatibility, biological properties, and toxicity in osteoblast-like cells.
Methods: Having developed the alloy ingots by induction melting, we used the cast rod as a plasma cathode in a filtered cathodic vacuum arc deposition chamber to coat a 25-nm thin film of amorphous alloy on cover glass slides. These coated cover glass slides were then examined for biocompatibility. The biocompatibility tests in SaOS2 osteoblast-like cells were performed using a methylthiazol tetrazolium assay and alizarin red staining. The medical grade Ti-6Al-4V alloys was studied in parallel as a control material.
Results: There was no statistically significant difference in number of living cells between all novel alloys compared with Ti-6Al-4V thin film. Alizarin red staining showed that all novel alloy thin film had significantly higher percentage area of calcification in comparison with Ti-6Al-4V thin film control ($P < 0.05$). In terms of calcification size, the Ti44Zr10Pd10Cu10Co19Ta7 and Ti44Zr10Pd10Cu14Co15Ta7 showed significantly greater calcification than the control ($P < 0.05$) while Ti44Zr10Pd10Cu6Co23Ta7 also demonstrated larger calcification in comparison with control but no statistical significance ($P = 0.27$).
Conclusion: The results indicated that all investigated Ti-based alloys were found to be non-cytotoxic and support differentiation of osteoblast-like cells.

Keywords: Titanium-based alloy, biocompatibility, toxicity, calcification.

Amorphous alloys or metallic glass is a new class of alloys which has gained wide attention due to their superior properties compared with the conventional crystalline alloys. Metallic glass formation is achieved by passing nucleation and growth of crystalline phases when the alloy is rapidly cooled from the molten liquid. [1] In general, metallic glass has superior strength, lower elastic modulus, better corrosion resistance, better wear resistance and unique processing capabilities compared with crystalline alloy counterparts. The unique properties of amorphous alloys have the potential to solve problems encountered by traditional orthopedic implant materials. [2, 3] Ti-based amorphous alloy is one of the most popular alloys because of its potential to be used as a biomaterial. However, many of the Ti-based amorphous alloys contains toxic element including Al, Ni, V and Be. These toxic elements can be released from the alloys and causes long-term health problem, for example, peripheral neuropathy, osteomalacia and Alzheimer's diseases. [4, 5] We tried to obtained better performance and safety of the Ti-base amorphous alloy by exploring new compositions. This study aimed to develop a novel biocompatible composition, toxin-free Ti-based amorphous alloy and to study the *in vitro* biocompatibility of this novel alloy using Ti-6Al-4V alloy as a reference material in human osteoblast-like

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Received: January 26, 2018
Revised: July 24, 2018
Accepted: September 25, 2018

cells. Microscopy was used to examine the biocompatibility and alloy characterization.

Materials and Methods

Ti-based alloy ingots with a nominal composition of Ti44Zr10Pd10Cu6+×Co23-×Ta7 (× = 0, 4, 8) were synthesized using arc-melting 6 elements of 99.9% or higher purity in a titanium-gettered argon atmosphere. Ti-6Al-4V alloy was used as a reference material. Cylindrical rod samples with a diameter of 5 mm and length of 20 mm were fabricated by copper mold casting. The cylindrical rods of 3 new alloy formula and Ti6-Al-4V as a reference material were then used as plasma cathodes in filtered cathodic vacuum arc (FCVA) deposition to make Ti-based thin film metallic glass which is coated on glass substrate with a diameter 1.5 cm. The Ti-based thin film on glass substrate was further employed for biocompatibility tests.

We performed biocompatibility test with human osteoblast-like cells (SaOS2) *in vitro*. Before every test, the coated discs were sterilized by autoclaving. SaOS-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). The medium was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 unit/ml penicillin, 100 mg/ml Streptomycin and 0.25 μg/ml amphoteracin B. Cells were maintained at 37 °C in 100% humidity and 5% CO_2. Confluent cells were detached using 0.25% trypsin with ethylene diamine tetraacetic acid and re-suspended in fresh culture medium. The media were changed every 2-3 days. Cells proliferation was determined by methylthiazol tetrazolium (MTT) assay. Cells were seeded on triplicate samples discs (n = 3) with a concentration of 50,000 cells/well in a 24-well plate. The assay was performed on day 3. After completing the culture period, the media was gently removed and the specimens were rinsed with phosphate-buffered saline (PBS) to remove unattached cells and to avoid the effects of media on the biochemical assays. Then MTT solution (300 μL; 0.5 mg/mL 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide in culture medium without phenol red) was added. After 30 minutes of incubation, MTT solution was discarded and then formazan crystals were dissolved in dimethylsulfoxide (DMSO) (900 μL/well) and glycine buffer (pH = 10) (125 μL/well). The absorbance was read at a wavelength of 570 nm by Thermospectronic Genesis 10 UV-vis spectrometer.

Alizarin is an organic compound that can react with calcium ions. Alizarin red S, a dye that stains calcium salts selectively and is widely used for mineral histochemistry of calcium, served to analyze the mineralization level of cells. In this study, SaOS2 1.0×10^5 cells were seeded onto the disc. The osteogenic inductions were induced after 24 hours. On day 28, the medium was discarded, then the cells on the discs were fixed with 95% ethanol for 10 minutes, and then rinsed several times with distilled water; 0.1% Alizarin red was added onto the disc and incubated at 37 °C for 30 minutes and rinsed several times with distilled water before proceeding to light microscope evaluation under 10x magnification.

Results

The SaOS2 cell proliferation was determined by MTT assay on day 3 and are shown in Figure 1. There was no statistically significant difference in number of living cells between all the novel alloys compared with thin film Ti-6Al-4V. Alizarin red staining in Ti-6Al-4V and novel metallic glass alloys, are shown in Figure 2. The Alizarin red staining assay showed that all novel alloy thin film had significantly higher percentage areas of calcification compared with the Ti-6Al-4V thin film control ($P < 0.05$). In terms of calcification size, the Ti44Zr10Pd10Cu 10Co19Ta7 and Ti44Zr10Pd10Cu14Co15Ta7 showed significantly greater calcification than the control ($P < 0.05$) while Ti44Zr10Pd10Cu6Co23Ta7 also demonstrated larger calcification compared with control but no statistical significance ($P = 0.27$).

Alizarin red staining was analyzed using Image J (color threshold set as RGB: 200, 120, 80) to quantify the calcium mineralization. The results are demonstrated in Table 1.

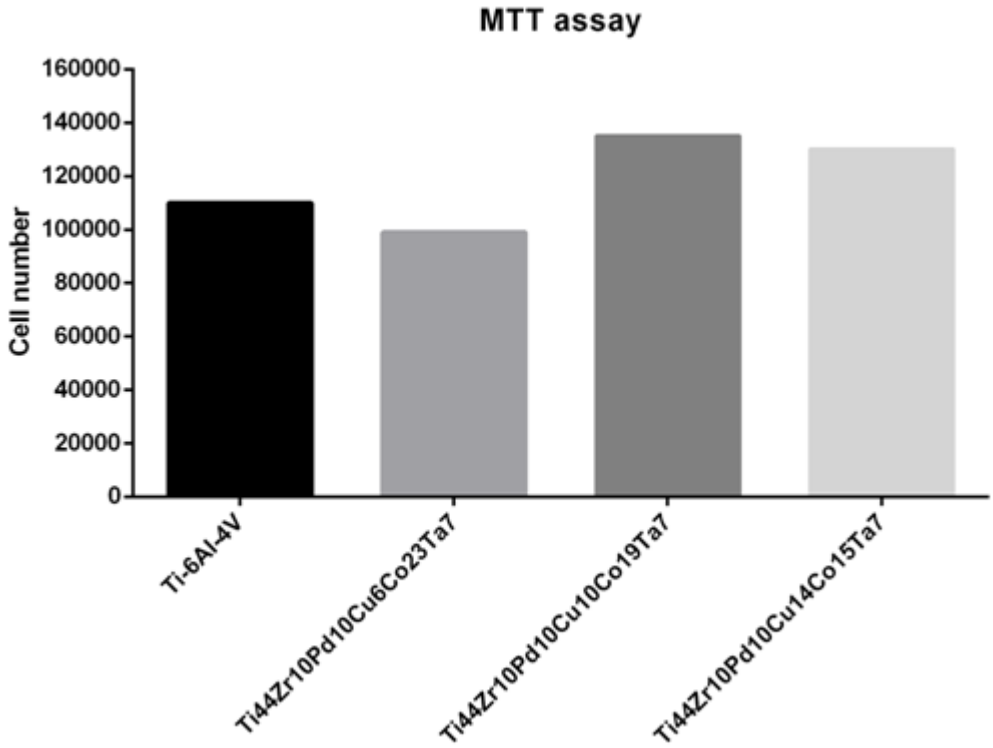

Figure 1. MTT assay of SaOS2 cells on Ti-6Al-4V and the series of novel Ti-based MG thin film.

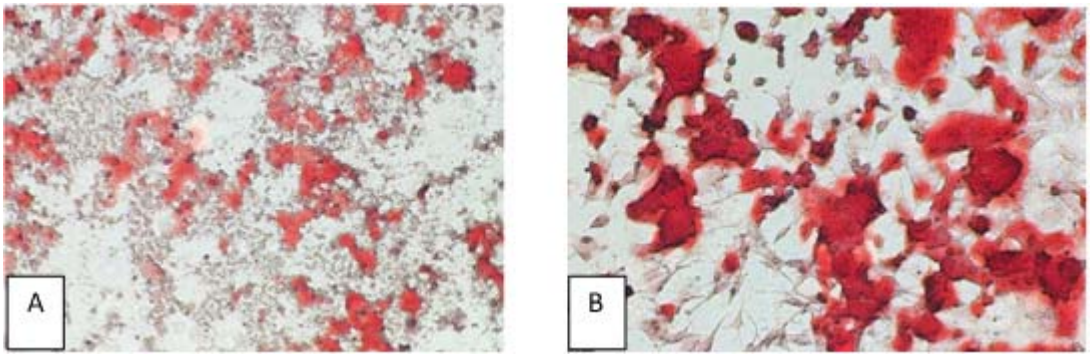

Figure 2. The Alizarin red staining results of SaOS2 cells on Ti-6Al-4V (Figure 2A) and the novel Ti44Zr10Pd10-Cu14Co15Ta7 thin film (Figure 2B).

Table 1. Quantitative analysis of Alizarin red staining using Image J.

	Ti6Al4V	**Ti44Zr10Pd10-Cu6Co23Ta7**	**Ti44Zr10Pd10-Cu10Co19Ta7**	**Ti44Zr10Pd10-Cu14Co15Ta7**
% Area	4.3%	7.1%	9.8%	18.6%
Average size (pixels)	96.3 ± 18.6	103.7 ± 5.3	310.5 ± 27.6	398.9 ± 106.5

Discussion

In this report, the new Ti-based alloy composite without toxic elements has been synthesized in the TiZrCuPd alloy system such as Ti40Zr10Cu36Pd14, which exhibit high corrosion resistance and good combination of strength and ductility, implying a high potential as biomaterials. [6] We developed our metallic glass based on this combination. We decided to decrease the copper composition due to the reported cytotoxicity of Cu to 3T3 fibroblast cells.[7] Cell toxicity of copper-containing crystalline alloy and amorphous alloy have been reported. Elshahawy WM, *et al.* [8] stated that Cu released from gold alloys, which are commonly used as fixed prosthodontic restorations, show evidence of a high cytotoxic effect on fibroblast cells. In contrast, a recent study did not demonstrated negative effects of copper-containing alloys on the cells [9] and they were compatible with the results of our study. The explanation of this issue is the formation of TiO2 that developed on the surface of novel Ti based alloy which are 44 atomic percentage Ti. The TiO2 has been reported to provide good biocompatibility and bactericidal effects. [10] It may conceal the copper from direct contact to the cells or decrease the copper ion release into the cell growth medium to the optimum level. In addition, our novel alloy compositions contain lower amounts of copper than the alloy previously reported which may lead to lower toxicity from copper. The results in our experiment are compatible with many previously published articles which focused on toxin free amorphous alloys containing copper composition. Qin FX, *et al*. [11] have developed an amorphous alloy with component of Ti40Zr10Cu40-×Pd10+× (with × = 0, 2, 4, 6, 8 and 10). They reported good mechanical properties and good biocompatibility of their series of amorphous alloy. Oak JJ, *et al*. conducted human osteoblast-like cells (SaOS2) on Ti45Zr10Pd10Cu31 Sn4 and found results of good biocompatibility and glass forming ability. [12 - 13] However, due to the nature of novel materials, it is impossible to find a previously matched amorphous alloy to study in comparison with the current study's results. In our study, after the cells were exposed to all the novel amorphous alloy samples for 3 days, there was no significant difference in cell proliferation and differentiation compared to the glass substrate and Ti-6Al-4V controls, suggesting that Ti-based MG thin film was non-toxic to SaOS2.

Conclusion

The novel Ti-based amorphous alloy Ti44Zr 10Pd10Cu6+×Co23-×Ta7 (× = 0, 4, 8) demonstrated biocompatible characteristics to osteoblast-like cells (SaOS2). These results suggest that the novel Ti-based amorphous alloy may be applied to potentially develop for using as biomedical applications.

Conflict of interest

None of the authors has any potential conflict of interest to disclose.

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