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Biocompatibility evaluation of Co-ribbons prepared by Melt-spinning

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Abstract
Ribbons of Co-base alloy were tested as biocompatible material at *in vitro* process to develop in the future an implant dental device. Regarding research on the effect of rapid solidification on Co-alloys, few or absent studies to date have been assessed for the evaluation of surfaces properties when the alloys are placed in contact with human mesenchyme stem cells (hMSC). The Co-based ribbons were prepared by rapid solidification process and its surface attached with human adipose-derived mesenchymal stem cells to determine it toxicity, cells viability, adhesive and proliferation of the cells. Experimental results showed that Co-based ribbons are non-toxic material with 80% of increased cellular adhesion and exhibited excellent biocompatibility.

Introduction
Metals alloys have been used as substitute biomaterials for missing tooth or bone tissues for thousands of years [1]. The studies have shown that alloys such as Ti-Al-V, Co-Cr-Mo and Co-Cr exhibit good biocompatibilities, osteoinductivity, osteoconductivity within tooth and bone tissues and adipose-derived stem cells [2, 3]. Traditional surgical biomedical applications of cobalt-base alloys over the past decades have been produced by traditional ingot metallurgy or wrought processing form that has shown compatible biological properties and good corrosion resistance in dental and bone tissue engineering [3-6]. Recently, a variety of methods as rapid solidification (RS) techniques [7-9], namely melt spinning, high-pressure gas atomization, laser powder micro deposition, electro beam melting, etc., have proved to be effective in improving the mechanical properties, the compositional homogeneity and microstructure of metals alloys. Such improvements in chemical homogeneity and microstructure offer the possibility of directly producing ribbons of Co-alloys at scales ranging from that of the laboratory to that of industry that can result in the refinement of both matrix grains and intermetallic particles, extension of solid solubility and improved chemical homogeneity and enhance particularly the durability [10, 11]. Melt spinning is one of such technique that enables the production of ribbon-shaped small-sized devices with improved ductility, transformation temperatures and hysteresis of metal alloys [12]. Earlier, our group described the effect of nanoprecipitates and grain size on the microstructure and on the mechanical properties of advanced structural steels by RS [13]. Many properties of melt-spun Co-ribbons alloys, including their microstructures, depend strongly on the processing parameters and a further characterizations are needed for evaluate their surfaces properties when the material is placed in contact with mesenchymal stem cells. The main objective of this research is to evaluate the surface characteristics of the Co-base alloys respect to the interaction between the biomedical implant prepared with the Co-base alloys and the living tissue to determine the biocompatibility process at *in vitro* cell culture.

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Materials and Methods

Co-ribbon alloy processing

Melt spun ribbons of Co-base alloy were obtained by rapid solidification technique. The Co alloy was melted in an alumina crucible by heating up by means of a copper coil connected to one Radyne unit operated at 10 kV. Once the alloy was melted, argon gas was injected on the top of the crucible with a pressure of 6 psi. The liquid melt was passed through a quartz tube nozzle with 1 mm in diameter and 5 mm in length and impinged onto a copper wheel that was rotating at 20 m/s to achieve cooling rate of 1 x 10^6 K/s for obtain ribbons with 40 cm of length, 3-4 mm wide and a thickness of 30 μm. Metallographic preparation of specimens was performed by using Struers electro polishing unit in a solution of 10 % perchloric acid in ethanol (20 volts at -10ºC). The prepared ribbons were chemically analyzed respect to composition by electron dispersion spectroscopy (EDS) resulting in a composition of: Co= 39.605 wt. %; Mn= 2.25 wt. %; Mo= 7.40 wt. %; Fe=15.90 wt. %; Ni= 14.30 wt. %; Cr= 20.40 wt. % and C= 0.145 wt. %. Surface microstructure observations were performed by using a Jeol STEM 1200 transmission electron microscope.

Biological Response

a) Cell Culture

Biological assays were performed using human adipose-derived mesenchymal stem cells line (ADSC) obtained from Invitrogen ADSC were cultured in 75 cm^2 cell culture flask in α-MEM supplemented with 10% fetal bovine serum, antibiotic solution (streptomycin 100 μg/ml and penicillin 100U/ml, Sigma Chem. Co) and 2 mM L-glutamin. For *in vitro* osteogenic differentiation to osteoblast lineage, ADSC cells were cultured in "osteogenic medium" consisting of complete α-MEM medium supplemented with 50 μg/mL of ascorbic acid, 10 mM glycerol-2-phosphate and 10^{-7} M of dexamethasone and we named as ADSC-OB. Passage between 2-4 were used for all the experimental procedures and incubated at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. All alloy where sterilized by incubating on 100% ethanol for 10 min and then washing with buffer of phosphates with antibiotic solution (streptomycin 100 μg/ml and penicillin 100U/ml, Sigma Chem. Co) and dry under the cell culture hood with UV by 30 min.

b) Cell attachment

The cell adhesion of ADSC and ADSC-OB onto cobalt-based alloys was evaluated using the vibrant cell adhesion assay kit (MolecularProbes). Briefly; cells cultures were incubated with calcein AM stock solution (5 μM) in serum-free medium for 30 min; washed with PBS, trypsinized and seeded at 1x10^4 cell/ml onto cobalt-based alloys and incubated for 4 h and 24 h. The fluorescence was quantified using a fluorescein filter set with a Wallac Victor^3 1420 spectrophotometer (Perkin-Elmer, Boston, MA). The percentage cell adhesion was obtained by dividing the corrected (background subtracted) fluorescence of adherent cells by the total corrected fluorescence of control cells and multiplying by 100%. Conventional polystyrene 24 well culture plates were used as a control.

c) MTT assay

Cell viability of ADSC and ADSC-OB plated at concentration of 1x10^4 cell/ml in triplicate onto cobalt-based alloys were checked by the MTT assay for 3, 5 and 7 days of culture. The ADSC and ADSC-OB seeded onto cobalt-based alloys at prescribed time were washed with PBS and incubated with fresh cultured medium containing 0.5 mg/mL of MTT for 4 h at 37°C in the dark. Then, the supernatant was removed and dimethyl sulfoxide (DMSO) was added to each well. After 60 minutes of slow shaking the absorbance was quantified by spectrophotometry at 570 nm with a plate reader. The culture medium during experimental time was changed every two days with fresh media.

d) Cell morphology

For cytoskeletal organization of the ADSC cultured onto cobalt-based alloys, the cells were seeded at a concentration of 1 × 10^3 cells/ml and incubated for 24 hours in α-MEM cultured medium. After 24 hours the samples were washed with PBS and fixed with 4% paraformaldehyde for 10 minutes at

room temperature (RT), permabilized with 0.2% Triton X-100 for 5 minutes, washed twice with PBS and incubated with α-actin antibody diluted 1:100 in 0.2% of bovine serum albumine (BSA)-PBS for 1 h at RT. The cells were then gently washed twice with 0.2% BSA-PBS and twice with PBS. Then, cells were incubated with FITC secondary antibody diluted 1:1000 in PBS for 1 hour. The cells were gentile washed with PBS and visualized by means of indirect immunofluorescence (Axiophot, Carl ZeissR, Germany).

e) Statistical Analysis

Statistics for experimental assay were performed with the Student's *t*-test, using Sigma Stat V 2.0 software (Jandel Scientific). Results of $p < 0.05$ values were considered significant to test onto cobalt-based alloys material against the control.

Results and Discussion

Microstructural Charaterization of Cobalt Melt Spun Ribbons

Cobalt alloys showed a main microstructure consisted of α-Co grains with grain size showed values between 0.7 to 2.5 μm with tangled dislocations and in matrix; it was observed the presence of spherical precipitates with 0.13 μm in diameter (shown by arrows, Fig. 1A). Electron diffraction patter taken in α-Co grains showed a lattice parameters a = 2.507 Å and c = 4.070 Å, that represent a controlled microstructural architecture of the melt spun ribbons of cobalt base alloy (Fig. 1B). These microstructural features illustrate the unique nature of RS fabrication of Co-base alloy products and the prospect of fine grains for structure manipulation that could allow an effective bone biocompatibility of the material [14, 15].

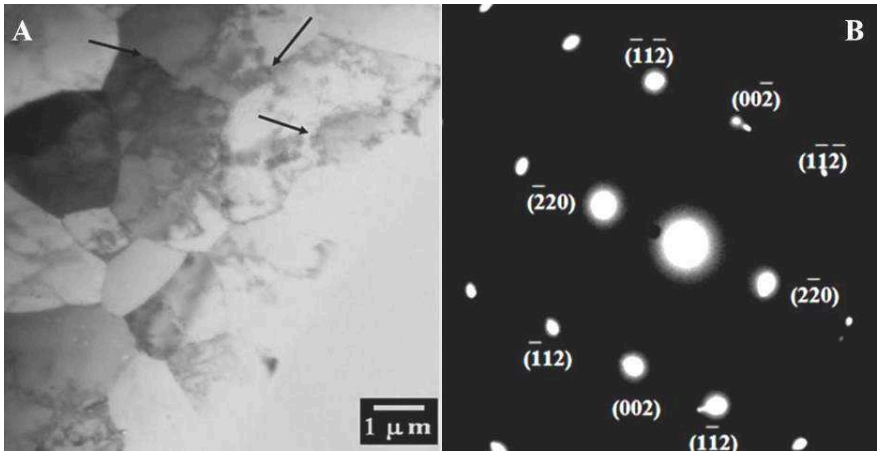

Figure 1. Microstructure of Cobalt melt spun ribbons where arrows show small spherical precipitates (A) and electron diffraction patterm of α-Co grains (B)

Biocompatibility Characterization

Cell adhesion on cobalt-based alloys was evaluated after 24 h of cell culture. Both cultures of ADSC and ADSC-OB showed a good cell-material interaction affinity for the surface alloy, resulting in comparable cell spreading morphology with multiple cellular extensions and filopodia over the material (Fig. 2A and 2B). The results of cell adhesion values after 4 and 24 h showed high values of attached cells. The cell adhesion as the first step to assess the compatibility of the ribbon surface in both cell cultures was greater on the cobalt-based alloy surface without any significant differences in term of adhesion between normal ADSC and differentiated ADSC-OB cells ($p < 0.05$). Therefore, should be noted that the adherent values at all-times were consistently higher (Fig. 2C).

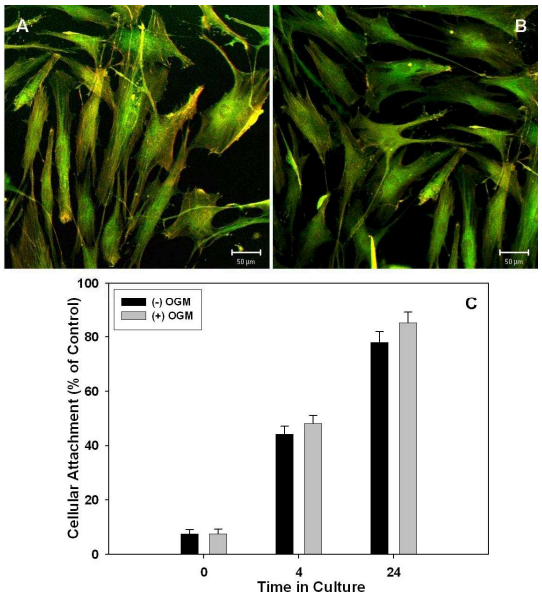

Figure 2. Cytoskeletal morphology of ADSC (A) and ADSC-OB (B) and cellular adhesion response C) when cultured onto the Cobalt melt spun ribbons.

Although, it is important to remark that increased cell attachment is a good indicator that the surface is not toxic to the cells; we perform the MTT assay to confirm the biocompatibility response. Our results showed high levels of MTT conversion and continue until day 7 for both ADSC and ADSC-OB (Fig. 3). The *in vitro* biocompatibility approach allows investigating in detail the interaction between cells and the surface material of Co-base alloys. This early phases is crucial; because the contact of the osteoprogenitor cells with the future implant surfaces is the basis for all the following events, including the deposition of an organized extracellular matrix and its mineralization [16-19].

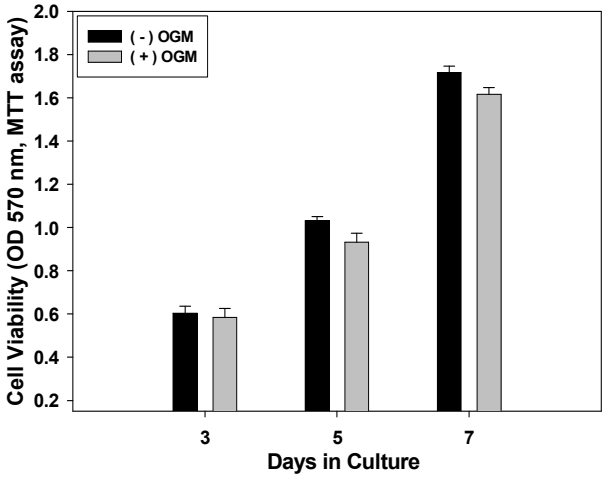

Figure 3. Cell viability of ADSC (-OGM) and ADSC-OB (+OGM) cultured onto the Cobalt melt spun ribbons.

Conclusions

From all results reported in this study we can conclude that these microstructural features in the melt-spinning cobalt-base alloys surface showed good biocompatibility and is a favorable material for dental implant device. Our preliminary results concerning cell biocompatibility; induce us to elaborate one future prospect of structure device for biomedical implant applications. However, more studies focus on the biomineralization process are needed for future bone tissue regeneration applications.

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