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IN VITRO STUDY ON BIOCOMPATIBILITY OF NEW POROUS NIZR ALLOY AS POTENTIAL BIOMATERIAL

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ABSTRACT

The objective of this work was in vitro testing on biocompatibility of a new NiZr alloy as potential biomaterial for reconstructive medicine. Flat tablets of porous NiZr alloy and non-porous zirconium were prepared by combustion synthesis technique. The cytotoxicity of materials was evaluated in KCL-22 (human chronic myeloid leukemia) suspension cell line with the vital dye (trypan blue) exclusion test. To test materials adhesiveness, HeLa monolayer cells previously stained with fluorescent dye were seeded on material surface, incubated for 24-48 h and examined under a fluorescence microscope. To analyze the cell growth rate, HeLa cells seeded on the tablet surface were incubated for 3-10 days, trypsinized and counted with haemocytometer. The materials were shown to be non-toxic and non-adhesive for cells; they can be classified as biologically inert. The same materials coated with collagen provided the cell attachment and spreading. The adhered cells expressed typical fibroblast-like morphology; they survived as long as 10 days and multiplied.

NiZr alloy tested is biocompatible and able to support cell adhesion, survival and growth. It may be regarded as a potential biomaterial for reconstructive medicine.

Keywords: biocompatibility, test-system in vitro, NiZr alloy, reconstructive medicine.

INTRODUCTION

In the last years, the intensity of research on implantable biomaterials and their application in reconstructive medicine rapidly increases [*Ratner B. et al., 2004*]. The requirements to these materials are numerous and diverse [*Williams D., 2008*]. Particularly, their mechanical properties should match those of the tissue at the site of implantation; materials should be biocompatible to match cell/tissue growth and should not provoke adverse host responses. Metals and metallic alloys are widely used and are indispensable as hard tissue (particularly, bone) substitutes. Due to high mechanical reliability, they cannot be replaced with ceramics or polymers [*Li J. et al., 2006; Ryan G. et al., 2006; Staiger M. et al., 2006*]. Metal bone substitutes are necessary to mimic the tissue architecture (to be highly porous with an interconnected pore network) to provide osteocyte ingrowth and flow transport of nutrients and metabolic wastes. The appropriate pore size is shown to be 100-500 μm [*RatnerB. et al., 2004*]. For the last decades, new biomaterials based on zirconium alloys attract attention, as they combine the proper balance of mechanical properties and corrosion resistance with high biocompatibility [*Hunter G., Mishra A., 2004; Good V. et al., 2005; Hunter G., Pawar V., 2005*].

In vitro studies are the starting point in evaluating new biomaterials biocompatibility. Cell cultures are a simplified system that minimizes the effect of confounding variables and allows evaluating basic toxicological profiles of new materials. In modern toxicological research, cytotoxicity *in vitro* testing becomes mandatory in safety assessment [*Gasparyan G. et al., 2009*] and is widely used to predict acute toxicity *in vivo* [*Gennari A. et al., 2004; Marx U., Sandig V., 2007*]. Cell cultures

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are also applied to study the broad spectrum of interactions between cells and biomaterials, so called bio-functionality of implantable materials (their ability to provide cell survival, adhesion, migration, proliferation, *etc.*) [*Kirkpatrick C. et al., 1998; Cheung S. et al., 2007*]. Investigations of such kind are of vital significance, as they allow predicting the *in vivo* healing response, potential outcomes of biomaterial implantation, and warning about possible adverse effects.

A current challenge in manufacturing intermetallic biomaterials is development of simple and non-traditional synthesis methods providing required characteristics of products. Recently, the combustion synthesis (CS) technique [*Munir Z. Anselmi-Tamburini U., 1989; Merzhanov A., 1996*] has been applied to produce novel biomaterials. Advantages of CS include low energy requirements, the simplicity of experimental device used, chemical homogeneity and high purity of the product.

Earlier we have applied the CS technique to synthesize novel NiZr intermetallic alloys as porous biomaterials [*Manukyan Kh. et al., 2010*]. The initial mixture contained Teflon $(C_2F_4)_n$ as an additive to facilitate pore formation. The alloys were tested *in vitro* for biocompatibility. It was shown that fabricated materials included water-soluble cytotoxic components, probably, fluoride derivatives. These compounds could be washed out by the aqueous solvents. The alloys tested were demonstrated to be not cytotoxic by themselves [*Manukyan Kh. et al., 2010*].

Recently we have fabricated new porous alloy excluding Teflon to avoid the toxicity of fluoride in the product (our unpublished data). The objective of this work was *in vitro* testing for biocompatibility of a new NiZr intermetallic alloy as potential biomaterial for reconstructive medicine.

MATERIALS AND METHODS

Biomaterial specimens tested: Rhombic flat tablets with the area about 1.0 cm^2 made of porous NiZr alloy (85% Zr – 15% Ni) and round flat tablets with the area about 0.8 cm^2 made of non-porous zirconium (Figure 1) were prepared by CS technique. The porosity of alloy was about 60-70% (Figure 2) and the pore sizes were optimal to mimic bone architecture (100-500 *mm* [*Ratner B. et al., 2004*]). Tablets were treated with 10 M NaOH and

Figure 1. *Rhombic tablet of NiZr alloy (left) and round tablet of Zr (right).*

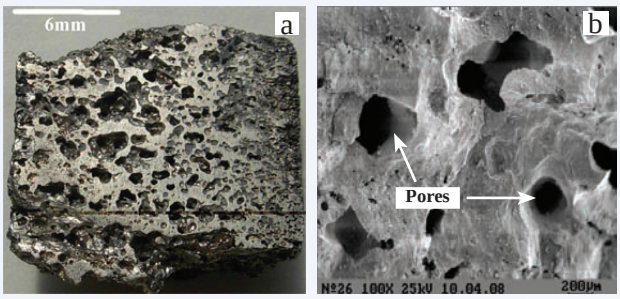

Figure 2. *The structure of porous NiZr alloy. a) photography; b) scanning electron microscopy.*

heated in a vacuum for 2 *h* to induce oxidation of the metal surface. Then a part of tablets were covered with hydroxyapatite, a mineral component of the bone tissue, by precipitation from a saturated solution. Prior to cell culture experiments, the tablets were cleaned in water and heat sterilized (180ºC for 2 *h*). The tablets were numbered for tracking purposes in the process of experiments.

Testing of materials cytotoxicity: The cell line used to test materials cytotoxicity was KCL-22 (human chronic myeloid leukemia cells, suspension culture). Cells were routinely maintained in the growth medium RPMI-1640 (Sigma–Aldrich, Germany) supplemented with 10% fetal bovine serum (Biochrom AG, Germany) and antibiotics at 37ºC in air atmosphere. Sterilized tablets of materials were placed into wells of 24-well plate (Corning), a tablet per a well. Cell suspension was added into wells (cell density 0.5 x 10^6 *cells/mL,* 1 *mL* of the cell suspension per a well) to cover the tablets (Figure 3). Cells were incubated for 48 *h* and the viable cell number was counted by the vital dye (trypan blue) exclusion test [*Strober W., 1997)*]. This test overestimates the number of viable cells, but it is quite applicable to comparative studies. Cell viability was expressed as a percentage of intact controls (cells grown in wells containing no material tablets). The experiments were performed in triplicate.

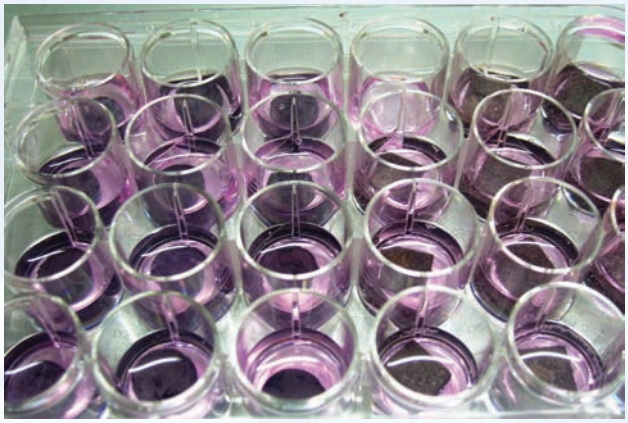

Figure 3. *Tablets of materials and KCL-22 cell suspension in the wells of a 24-well multiwell plate.*

Testing of materials adhesiveness for cells: The cell line used was HeLa (human cervix carcinoma cells, monolayer culture). Cells were routinely maintained in the growth medium DMEM (Sigma–Aldrich, Germany) supplemented with 10% fetal bovine serum (Biochrom AG, Germany) and antibiotics at 37ºC. Sterilized tablets, uncovered or covered by hydroxyapatite and/or collagen, were placed into glass 15 *mL* vials. The collagen solution was prepared from rat tail tendons dissolved in 3% acetic acid for 24 *h* at continuous agitation and then centrifuged to discard undissolved tissue pieces. To cover the tablets with collagen, they were immersed into collagen solution and air dried. This procedure was repeated two times, and the surface of tablets was sterilized by UV-radiation. To make visible the cells on the surface of non-transparent tablets, they were previously stained with fluorescent dye. For this purpose, confluent cell monolayers were incubated with Acridine orange (AO) (Sigma–Aldrich, Germany) at concentration 10 *mg/mL,* pH 4.2-4.3, for 30 *min* and washed with PBS two times. Then the cells were trypsinized (with trypsin-EDTA solution, Sigma–Aldrich, Germany), cells suspension was poured into the flasks over the tablets (cell density 0.25 x 10^6 *cells/mL*, 2 *mL* of cell suspension per vial) and incubated for 24-48 *h*. Then the tablets were extracted from vials and examined for attached cells under a fluorescence microscope Zeiss III RS (Germany) equipped with 560 *nm* excitation filter, 530 *nm* barrier filter and a CCD video camera PCO (Germany). The program ISIS-1 was used to record the cells attached on the materials surface. AO stained cells grown on the glass coverslips were used as a control.

Analysis of the cell growth rate: Heat sterilized tablets of tested materials were placed into 15 *mL* glass vials and suspension of trypsinized HeLa cells was added into the vials (cell density 0.3 x 10^6 *cells/mL,* 2 *mL* of cell suspension per a vial). After 3, 7 and 10 days of incubation the tablets were taken from the vials, washed with PBS to discard non-attached cells and treated with trypsin-EDTA solution. The obtained cell suspension was mixed with trypan blue, and the number of living cells was counted with haemocytometer.

Statistical treatment: The results obtained were statistically treated with Student's one-tail *t*-test.

RESULTS

Materials cytotoxicity: The materials tested did not express cytotoxicity (Figure 4). The variations in number of living cells after 48 *h* incubation with materials were statistically non-confident in comparison with the control. It can be concluded that materials tested did not contain any extractables harmful for the cells and did not suppress the cell viability at direct cell–material contact.

Materials adhesiveness for cells: Both non-porous tablets made of zirconium and porous ones of NiZr alloy, uncovered and covered with hydroxyapatite, were revealed to be non-adhesive for cultured cells. Microscopic examination of surface of tablets incubated with cells for 24-48 *h* after cell seeding did not reveal any cell attached. Hence, the materials tested, being non-toxic and non-adhesive for cells, can be classified as biologically inert and having no biological activity [*Williams D.,*

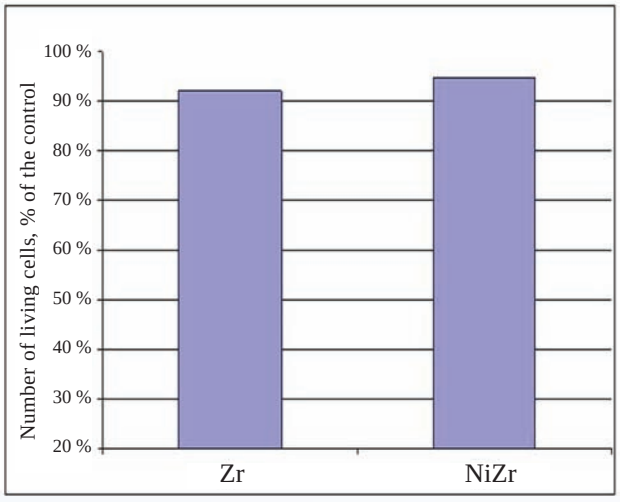

Figure 4. *The cytotoxicity of materials tested for KCL-22 cell line. Y-axis. Number of living cells, % of the control. X-axis. Zr; NiZr.*

2008]. Chemical and biological inertness is of key importance for biomaterials not to harm adjacent tissues at the site of implantation. The same materials coated with collagen provided the cell attachment and spreading. The adhered cells expressed typical fibroblast-like morphology similar to that of cells attached to glass surface (Figure 5).

Cells long-term viability and growth on the surface of materials: Cells grown on collagen coating showed about 100% viability after 3, 7 and 10 days of culture (Figure 6). The number of cells continuously increased from 3 to 10 days of culture incubation. Thus, being covered by collagen as the main component of extracellular matrix and bone, the tested materials provided not only cell adhesion but also cell survival and multiplication.

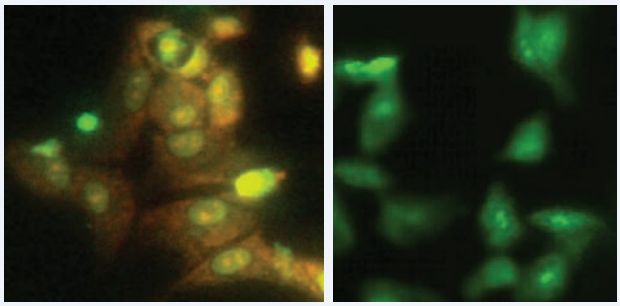

Figure 5. *HeLa cell line grown on glass (left) and on NiZr alloy coated with collagen (right).*

DISCUSSION

The safe evaluation of biocompatibility of metallic-based biomaterials is a prerequisite for their use in medicine. Application of *in vitro* models in this context is of growing importance as alternative (non-animal) methods [*Sabbioni E. et al., 2005*]. In spite of a plethora of possible test methods, the protocols currently required by international regulatory agencies for the evaluation of biocompatibility (direct or indirect contact assay, or adding a diluted extract from the biomaterial to the culture medium [*Wallin R., Arscott E., 1998; ISO 10993-5: 2009(E)*]) do not incorporate quantitative tests.

The recommended qualitative assessments involve estimation of acute toxicity through the gross microscopic examination of tissue; so, the results can obviously rely on the subjective expertise of investigators in identifying cell morphology and the severity of the reaction [*Marois Y. et al., 1996*].

In order to quantify the assay, in the present work the cytotoxicity of materials was evaluated by quantitative vital dye exclusion test. It was shown that the materials tested did not contain soluble toxic components and did not induce cell death at direct contact. Low chemical activity is considered to be the most important requirement to biomaterials intended for long-term contact with tissues of the human body not to cause harm to that body and provide co-existence of the implant with surrounding tissues [*Williams D., 2008*].

Artificial substitutes of such a structurally complex tissue as the bone should obviously mimic its architecture. The NiZr alloy used in the present work was a lightweight, highly (60-70%) porous material with interconnected pore network; pore sizes were comparable with those of the bone. Structurally, this material seems to be able to serve as scaffolding for bone cells to guide and to encourage *de novo* tissue formation.

To facilitate the cell in growth, the surface of biomaterials is known to be adhesive for cells. According to our results, the materials tested did not possess this activity. Coating of materials with hydroxyapatite, the major solid component of human bone, was not sufficient to provide cell attachment. This result seems to be contradictory to the known data on adhesiveness of hydroxyapatite for cells [*Mehta J. et al., 2005; Ma X. et al., 2007*]. In this context it is reasonable to notice that the attachment and spreading of cells is known to depend on specific biogenic proteins mediating the adhesive interaction between cells and inorganic substrata. These proteins may be of extracellular origin (e.g., from serum [*Okamoto K. et al., 1998*] or extracellular flu-

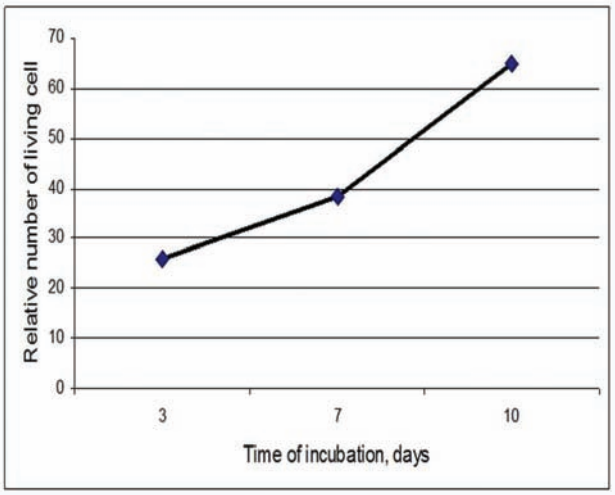

Figure 6. *The growth of HeLa cell line on the surface of NiZr alloy coated with collagen. Y-axis. Relative number of living cells. X-axis. Time of incubation, days.*

ids of the organism) and/or can be deposited by cells as an extracellular matrix [*Wu X. et al., 1998*]. In our experiments, collagen, another main bone constituent and the most abundant component of the extracellular matrix, was demonstrated to be an excellent substrate for cells to attach and spread. This effect of collagen was independent on the presence/absence of hydroxyapatite undercoating.

There are literature data [*Hsu F. et al., 1999; Wahl D., Czernuszka J., 2006*] on efficiency of hydroxyapatite-collagen composites as biomaterials for hard tissue repair. The presence of hydroxyapatite is proposed to favor the osseointegration, i.e. direct structural and functional connection between living bone and the surface of the implant [*Neo M. et al., 1992 a, b*]. As to the cell interaction with the composites mentioned, it is probably mediated by collagen, an adhesive biogenic molecule. It would be of interest to study the adhesive behavior of cells on metal materials coated with other components of the extracellular matrix [*Larsson R. et al., 1989; Buttafoco L. et al., 2006*].

In our observations, the appearance of HeLa cells spread on collagen coating was typical for fibroblastic, epithelial or endothelial cells grown of subcofluent cultures (so called "fibroblast-like morphology"). As the cell morphology regulates multiple aspects of cell behavior (survival, growth, differentiation, *etc.*), this fact suggested integrity and functional activity of the cells. Really, the attached cells were shown to survive for long time (to 10 days) and multiply. In future we propose to investigate other interactions between cells and porous NiZr alloy.

Thus, the NiZr alloy tested is biocompatible and able to support cell adhesion, survival and growth. It might be regarded as a potential biomaterial for reconstructive medicine.

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