Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. 80 (12) 1541–1552 (2015)
JSCS–4818
UDC 546.73'76'77–034:66.017:577.11+
57.001.365:576+615.9:616.314
Original scientific paper

In vitro biocompatibility assessment of Co–Cr–Mo dental cast alloy

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(Received 5 May, revised 13 August, accepted 26 August 2015)

Abstract: Metallic materials, such as Co–Cr–Mo alloys, are exposed to aggressive conditions in the oral cavity that represents an ideal environment for metallic ion release and biodegradation. The metallic ions released from dental materials can cause local and/or systemic adverse effects in the human body. Therefore, dental materials are required to possess appropriate mechanical, physical, chemical and biological properties. The biocompatibility of metallic materials is very important for dental applications. Accordingly, the aim of this study was to examine metallic ion release and cytotoxicity of Co–30Cr–5Mo cast alloy as the initial phase of biocompatibility evaluation. Determination of the viability of human (MRC-5) and animal (L929) fibroblast cells were conducted using three *in vitro* test methods: the colorimetric methyl-thiazol-tetrazolium (MTT) test, the dye exclusion test (DET) and the agar diffusion test (ADT). Furthermore, the morphology and growth of the cells were analyzed using scanning electron microscopy (SEM). The obtained results indicated that Co–30Cr–5Mo alloy did not release harmful elements in concentrations high enough to have detrimental effects on human and animal fibroblasts under the given experimental conditions. Moreover, the fibroblast cells showed good adhesion on the surface of the Co–30Cr–5Mo alloy. Therefore, it could be concluded that Co–30Cr–5Mo alloy is a biocompatible material that could be safely used in dentistry.

Keywords: Co-based alloy, biomaterials, cytotoxicity, fibroblasts.

INTRODUCTION

The most extensively used metallic materials in dental practice are commercially pure titanium (CPTi), and titanium- and cobalt-based alloys, whilst

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doi: 10.2298/JSC150505070M

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stainless steels were abandoned primarily due to nickel-induced hypersensitivity of the organism.[1] CPTi and its alloys are mostly used as endosseous implants, while Co–Cr–Mo alloys have been widely applied as subperiosteal implants and removable partial denture frameworks due to their outstanding mechanical properties and high corrosion resistance.[2,3] In fact, Co–Cr–Mo alloys have better mechanical strength and corrosion resistance compared to stainless steel.[4] Chromium, the main alloying element in Co–Cr–Mo alloys, is added to advance the formation of a stable passive oxide layer that contributes to corrosion resistance, while molybdenum is also frequently added to increase alloy resistance to pitting and crevice corrosion. The composition of Cr and Mo in commercial alloys lies within the range of 11–25 mass % and the corrosion behaviour of Co–Cr–Mo alloys depends primarily on the Cr and Mo levels in the alloy, *i.e.*, alloys with lower amounts of Cr and Mo are found to be more susceptible to corrosion.[5] Although the metallic dental materials are considered to have excellent corrosion resistance, numerous studies showed that metallic ions could be released into the surrounding environment.[6,7] The released metallic ions from dental materials could diffuse into mucosal tissue or could be distributed throughout the human body and cause adverse biological effects, depending on the ion type and concentration.[4,8] For instance, Ichinose *et al.*[9] showed that Co–Cr–Mo alloys disintegrate easily in cells, *i.e.*, Co dissolves from the peripheral areas of the cells although Cr remains within them. The main factors affecting metallic ion release from dental materials are the quantity and quality of the saliva, plaque, pH value, temperature and presence of proteins. Additionally, the physical and chemical properties of food and liquids as well as oral health conditions have great influences on ion release.[10] Furthermore, the composition and pre-treatment of the materials are very important factors that have a significant influence on metallic ion release.[11,12] Consequently, the contact between Co–Cr–Mo alloys and human saliva leads to the release of metallic ions.[13,14] It should be emphasized that biocorrosion of Co–Cr–Mo alloys is one of the major problems during their application as dental materials.[4] Many authors reported toxic and carcinogenic effects induced when humans and animals are exposed to certain metals.[11] Thus, the requirements of dental materials are primarily non-toxicity and biocompatibility. It should be underlined that biocompatibility of metallic materials is dependent on the release of elements from the materials.[12] Therefore, the main purpose of this study was to examine metallic ion release in artificial saliva and *in vitro* cytotoxicity of Co–30Cr–5Mo alloy. The cytotoxicity of the mentioned alloy was assessed using human (MRC-5) and animal (L929) fibroblast cell lines according to ISO 10993-5 and ISO 7405 standards, respectively.[15,16] The effects of Co–30Cr–5Mo cast alloy on cell viability, morphology and spreading on the surface were also determined in this study. Determination of cells viability was conducted using three *in vitro* test methods: the colorimetric methyl-thiazol-

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-tetrazolium (MTT) test, the dye exclusion test (DET) and the agar diffusion test (ADT). Although only the ADT assay has been prescribed in ISO 7405 standard,[17] the use of different test methods is highly advisable.[18] For the purpose of cells morphology and spreading analyses, scanning electron microscopy (SEM) was used. It is very important to mention that the increased worldwide interest in utilizing Co-based alloys for dental applications is related to their low cost and adequate mechanical properties,[3] and therefore Co–30Cr–5Mo alloy was chosen for examination in this study.

MATERIALS AND METHODS

Material preparation

The chemical composition of Co–30Cr–5Mo alloy (Wironit® extra-hard, Bego, Germany) used in this study was (in mass %): Co 63.0, Cr 30.0, Mo 5.0, max. 0.4 C, and trace amounts of Si and Mn. The physicochemical properties of the examined alloy were presented in a previous study.[13] The Co–30Cr–5Mo alloy in the as-cast condition was selected for consideration in this study for two reasons: 1) Co-based alloys are most often used in cast or cast and annealed metallurgic conditions[2] and 2) this type of alloy is widely used in dental practice, mostly for the manufacture of crowns, bridges and denture bases,[19] regardless of some results which indicate that harmful effects were induced by released ions and cells damage were caused by Co–Cr–Mo alloys.[1,9] The cylindrically-shaped specimens (8.0 mm in diameter and 15.8 mm in height) were cut into disc-shaped samples (8.0 mm in diameter and 4.0 mm in thickness). Subsequently, the samples were ground to 1200 grit with silicon carbide (SiC) papers and polished using diamond paste. Thereafter, the samples were cleaned in an ultrasonic bath with ethanol for 15 min followed by rinsing with distilled water for 5 min in order to eliminate surface impurities.

Microstructure characterization and hardness determination

The microstructure characterization of Co–30Cr–5Mo alloy used in this study was realized using a Carl Zeiss Opton Axioplan optical microscope (OM) and a JEOL JSM 5800 scanning electron microscope (SEM), which was operated at an accelerating voltage of 25 keV. Before the microscopic analysis, the examined material was etched using a solution containing 92mL HCl, 5mL H_2SO_4 and 3mL HNO_3. The Vickers hardness, HV, was measured on the polished mirror-like surface of the samples using a Buehler Identamet microindentation hardness tester, model 1114, under a load of 2.94 kN for 5 s.

Metallic ion release

After standard metallographic preparation of the samples and their ultrasonic cleaning, each sample was placed in a separate glass test tube with 5 mL of artificial saliva (Helvepharm AG, Switzerland) and thermostated at 37 °C. In order to designate the effect of the pH value of the artificial saliva on metallic ion release from Co–30Cr–5Mo alloy, the pH value was set to different levels (7.5, 5.5 and 4.0). The concentrations of released metallic ions were quantified after 1, 3 and 6 weeks using an Agilent ICP MS 7500ce inductively coupled plasma-mass spectrophotometer (ICP-MS).

Cell lines

The human (MRC-5) and animal (L929) fibroblast cell lines were grown attached to the surface of flasks (Costar, 25 cm^3) in Eagle's medium modified by Dulbecco (DMEM, Gibco

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BRL, UK) with 4.5 g L^{-1} glucose and 10 % foetal calf serum, FCS (Sigma). The medium contained the antibiotics penicillin 100 IU mL^{-1} and streptomycin 100 μg mL^{-1}. The cell lines were maintained under standard conditions at 37 °C under 5 % CO_2 humidified environment (Heraeus). The cell lines were transplanted twice weekly, and the logarithmic phase of growth between the third and tenth transplantation was used in the assays. The cell number and percentage of viable cells were determined by the colour test rejection with 0.1 % trypan blue. The viability of cells used in the assays was over 90 %.

Cell morphology

For the purpose of the morphological characterization of MRC-5 cells on the surface of Co–30Cr–5Mo alloy, the cells were collected during the logarithmic phase of growth, trypsinized, resuspended and counted in 0.1 % trypan blue. Subsequently, the cells (1×10^5 cells mL^{-1}) were seeded directly on the material surface and cultivated at 37 °C under a 5 % CO_2 humidified environment for 48 h. After the incubation period, the MRC-5 cells were photographed with a Canon 1100D camera attached to an inverted microscope Reichert–Jung Biostar 1820 E with 20 and 40× magnification objectives. SEM analysis of MRC-5 cells was performed on the same sample using SEM MIRA3 Tescan operated at an accelerating voltage of 20 keV. Before the SEM observations, the MRC-5 cells were fixed in 2.5 % glutaraldehyde for 48 h and dehydrated using the following solutions: 3 % acetic acid, 3 % acetic acid and 25 % ethanol at a ratio 1:1, 3 % acetic acid and 50 % ethanol at a ratio 1:1, and 70 % ethanol. Subsequently, the MRC-5 cells were coated with a thin Au–Pd layer using a Baltec SCD 005 sputter coater.

In vitro *cytotoxicity tests*

The Co–30Cr–5Mo alloy cytotoxicity was measured as the percentage of cell growth inhibition using three types of *in vitro* tests: the colorimetric methyl-thiazol-tetrazolium (MTT) test, the dye exclusion test (DET) and the agar diffusion test (ADT), which are briefly described in the Supplementary material to this paper.

RESULTS AND DISCUSSION

Microstructure characterization and hardness determination

The OM and SEM micrographs of Co–30Cr–5Mo alloy in as-cast condition are shown in Fig. 1. The microstructure of examined material consisted of

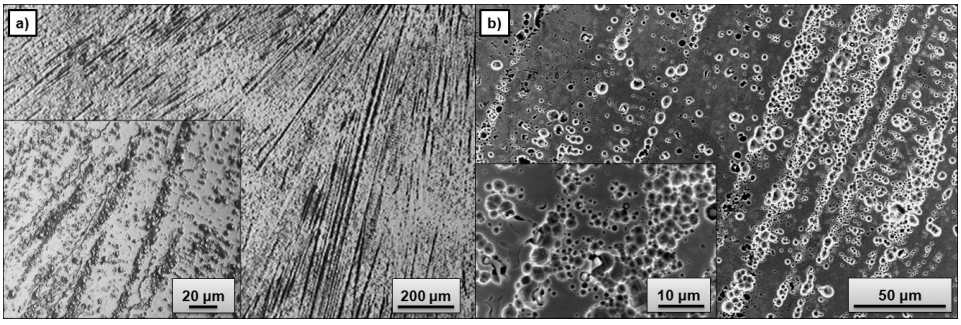

Fig. 1. a) OM and b) SEM micrographs showing the microstructure of the Co–30Cr–5Mo alloy in as-cast condition.

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dendrites (dark parts in Fig. 1a; light parts in Fig. 1b) and interdendritic regions (light parts in Fig. 1a; dark parts in Fig. 1b).

Similarly, Xin *et al.*[20] showed that the microstructure of a Co–Cr–Mo alloy obtained by traditional casting has a typical dendritic microstructure. Likewise, Patel *et al.*[21] described the microstructure of a Co–Cr–Mo alloy in details; the microstructure consisted of a solid Co matrix with interdendritic phases and carbides. The carbides were a combination of carbon and either Co, Cr or Mo, and were denoted as M_nC_n where M is Co, Cr or Mo. Furthermore, XRD analysis performed by the same authors indicated that the Co–Cr–Mo alloy (ASTM F75) consisted of face-centred cubic (fcc) Co, $M_{23}C_6$ and a sigma (σ) phase. It should be mentioned that the allotropic phase transformation of pure Co from the high temperature α phase (fcc) to the low temperature ε phase (hexagonal close-packed, hcp) occurs at about 420 °C.[22,23] Alloying elements, such as Fe and Ni, can stabilize the α phase, while Cr and Mo tend to stabilize the ε phase. Furthermore, Saji and Choe[22] using EDS analysis showed that the chemical compositions of the dendrites and interdendritic regions are similar. However, the dendritic regions are slightly rich in Cr and poor in Co. The obtained hardness of the Co–30Cr–5Mo alloy investigated in this study was 287.6±36.7 HV. According to Patel *et al.*,[21] the hardness of a Co–Cr–Mo alloy is correlated with its carbide content.

Metallic ion release

The metallic ion release testing in artificial saliva was preceded in this study by an *in vitro* cytotoxicity examination. As mentioned earlier, Co–Cr–Mo alloys are usually used to fabricate dental prostheses and subperiosteal implants, which are in contact with gingiva, and therefore artificial saliva was used as the testing solution. The obtained results are shown in Fig. 2.

As can be seen from the diagrams, the concentrations of released metallic ion increased almost linearly with increasing immersion time, a conclusion that was also reached by Nejatidanesh *et al.*[24] Furthermore, the metallic ion release rate increased with decreasing pH value of the artificial saliva. The most pronounced effect of the pH value of the artificial saliva on metallic ion release could be observed in the case of Co after 6 weeks of immersion. Similarly, Denizoglu *et al.*[25] investigated ion release from a Co-based alloy (alloy composition: Co 64.0, Cr 28.65, Mo 5.0, Mn 1.0, Si 1.0, C 0.35) and showed that the pH value of the testing solution significantly affected the total and Co ion release, but not Cr ion release. The concentrations of the released Cr and Mo did not differ drastically, but the weight content of Mo in the Co–Cr–Mo alloy was much smaller than that of Cr. Therefore, it could be concluded that the concentrations of released metallic ions do not reflect their weight contents in the alloy.[13,25] Many authors examined the corrosion resistance of Co–Cr–Mo alloys

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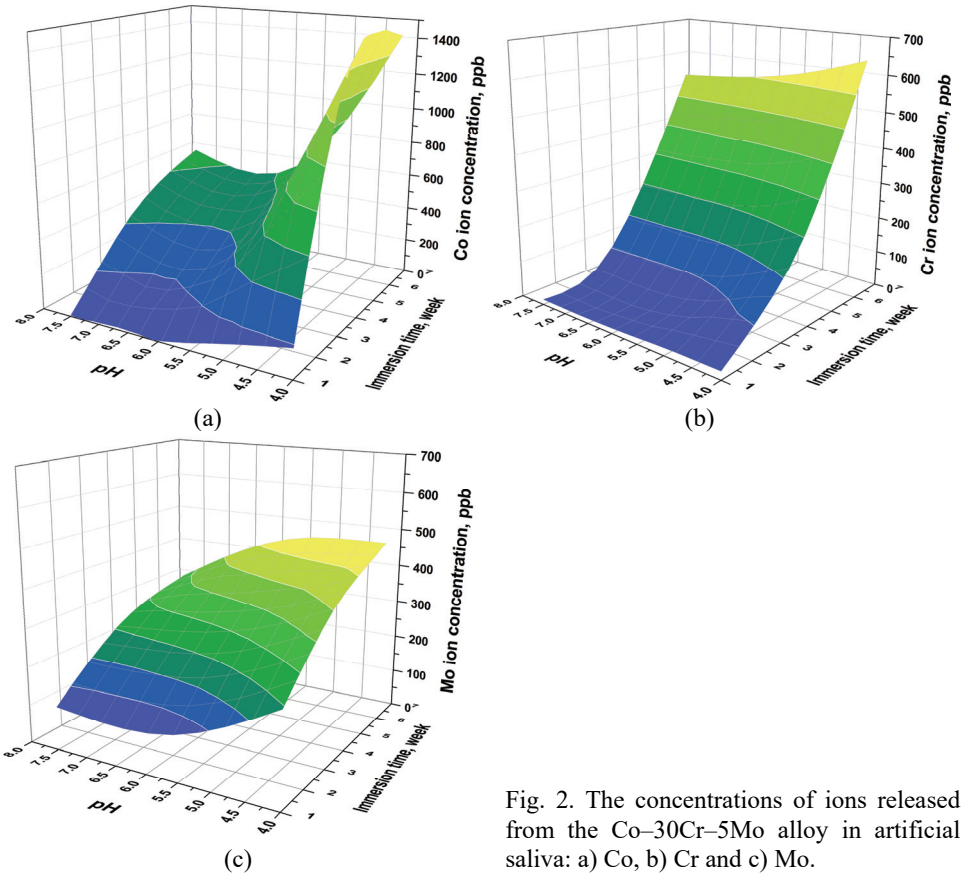

Fig. 2. The concentrations of ions released from the Co–30Cr–5Mo alloy in artificial saliva: a) Co, b) Cr and c) Mo.

and metallic ion release as a direct consequence of corrosion. For instance, Branzoi *et al.*[14] showed that the corrosion resistance of a Co–Cr–Mo alloy was higher than in the case of a Co–Cr–Ti alloy because the stability of the passive oxide film present on the Co–Cr–Mo surface was higher. Jevremović *et al.*[18] examined the release of ions from a Co-based alloy fabricated using both traditional casting and selective laser melting (SLM) techniques and concluded that the ion release rate was lower in the case of the SLM prepared alloy. Furthermore, Doni *et al.*[26] presented that a Co–Cr–Mo alloy showed a lower tendency to corrosion in a NaCl solution under sliding compared to the Ti–6Al–4V alloy. Puškar *et al.*[27] examined the behaviour of a Co–Cr–Mo alloy in artificial saliva at 37 °C for 7 days and concluded that the quantities of released Co, Cr and Mo were far below the permitted levels. According to the ISO 22674 standard, the quantity of an element released from the alloy should not exceed 200 μg cm^{-2} during 7 days.[28] In this study, Mo had the highest release rate after 7 days, but the amounts of the released ions never exceeded 2 μg cm^{-2}. Thus, the quantities

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of released ions were 100-fold lower than the permitted quantities. It is important to note that Beer-Lech and Surowska[29] showed that a Co–Cr–Mo alloy had very good passivation ability. Many authors linked metallic ion release with biocompatibility of dental materials.[24,25,30] It was observed that the concentration of released ions from the examined Co-based alloy was significantly higher when compared to other Co–Cr–Mo alloys[13] and it should therefore provide more information about the cytotoxicity potential of this alloy and the possibility of using an untreated Co–Cr–Mo cast alloy for dental applications.

In vitro *cytotoxicity tests as the initial phase of biocompatibility examination*

The results of the colorimetric methyl-tiasol-tetrazolium (MTT) and dye exclusion test (DET) assays, presented in Fig. 3, showed that the Co–30Cr–5Mo alloy did not exhibit cytotoxic effect either in contact with MRC-5 or L929 fibroblast cells. In fact, the results of the MTT test (Fig. 3a) indicate a gradual increase in cell viability with increasing contact time. After 72 h, both MRC-5 and L929 cells in contact with the Co–30Cr–5Mo alloy showed almost the same viability as the cells in the control sample. After 96 h, the cell viability further increased, meaning that the Co–30Cr–5Mo alloy did not exhibit toxic effects on the cells. The diagram of the DET results (Fig. 3b) shows an enhancement in cell viability with increasing contact time. As can be seen, the Co–30Cr–5Mo alloy showed excellent cytocompatibility with MRC-5 cells after 96 h, whilst the L-929 cells in contact with Co–30Cr–5Mo alloy had slightly lower survival rates than the MRC-5 cells. These differences, caused by the cell type which was used for testing, are negligibly small. Furthermore, since the decolourization index was 0 (no decolourization detectable around or under the disc-shaped samples) and the lysis index was 0 (no cell lysis detectable), the examined material was

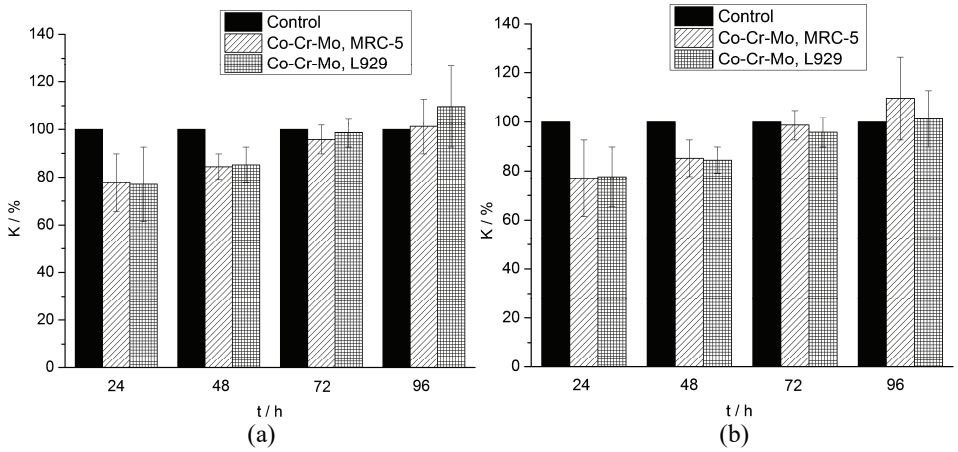

Fig. 3. Fractions of surviving fibroblast cells compared with the respective control (*K*) in: a) the MTT and b) the DET assay.

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not cytotoxic according to agar diffusion test (ADT).

These results are in accordance with the observation of Jevremović *et al.*[18] who demonstrated that an ASTM F75 Co-based alloy obtained by casting and SLM did not release harmful elements that could cause acute effects against animal fibroblast cells (L929). Similarly, Xin *et al.*[20] showed good spreading of mouse fibroblasts (3T3) on the surface of a Co-based cast alloy. The advantage of present study compared to studies by Jevremović *et al.*[18] and Xin *et al.*[20] is that the cytotoxicity of the Co–30Cr–5Mo alloy was examined using both human and animal fibroblast cells, because the use of human cells provides more valid results and better insight into the behaviour of this alloy in the human body. Different and often contradictory studies regarding cytotoxicity/cytocompatibility of Co–Cr–Mo alloys can be found in the literature. On the one hand, Čairović *et al.*[31] emphasized that cells adapted to the presence of a Co-based alloy after an initial toxic effect. On the other hand, it was shown that CPTi, Ti–Al–V and Co–Cr–Mo alloys caused cell damage in direct contact with cells, while in indirect contact, only the Co–Cr–Mo alloy caused cell damage.[1] Furthermore, Fleury *et al.*[32] demonstrated that Cr^{3+} (0–150 ppm) and Co^{2+} (0–10 ppm) ions have a cytotoxic effect on oesteoblast-like cells (MG-63). Microscopic analysis demonstrated changes in the shape, size and number of cells, whereas Co^{2+} had a greater effect on these parameters than Cr^{3+}. Even if there are articles in the literature that highlighted the cytotoxicity of Cr and Co, the results in this study indicated that the Co–30Cr–5Mo alloy did not exhibit cytotoxic effects and these results are similar to some published data.[18,20,31,33]

Cells morphology and adhesion

The photograph of MRC-5 cells in culture and in contact with the Co–30Cr––5Mo alloy is shown in Fig. 4. It can be clearly seen that the cells are attached to the edge of the disc-shaped sample of the Co–30Cr–5Mo alloy and that cells are mutually connected.

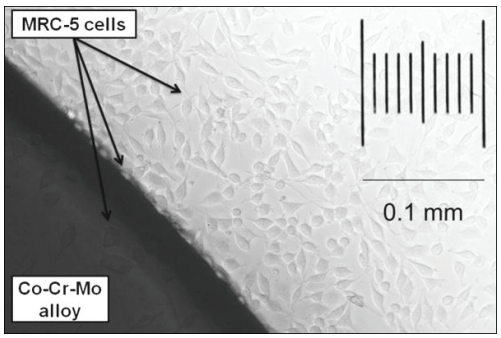

Fig. 4. MRC-5 cells in contact with Co–30Cr–5Mo alloy surface.

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The SEM micrographs of MRC-5 cells on the surface of the Co–30Cr–5Mo alloy are presented in Fig. 5. MRC-5 cells can have different shapes: triangle, spindle, elongated, oval, and flat[34,35] and in this study, the cells were rounded (Fig. 5a) and spindle elongated (Fig. 5b). The rounded cells were slightly smaller than spindle elongated cells, but they are very well spread on the Co–30Cr–5Mo alloy surface. Furthermore, these micrographs revealed the voluminous nature of the cells, which indicates that the cells are metabolically active. Excellent cell spreading is shown in Fig. 5c, which demonstrates that Co–30Cr–5Mo alloy is not harmful to the appearance of MRC-5 cells. It is obvious that the MRC-5 cells show good adhesion on the Co–30Cr–5Mo alloy surface, as can be seen in Fig. 5d, and thus the biocompatibility of the alloy was demonstrated.

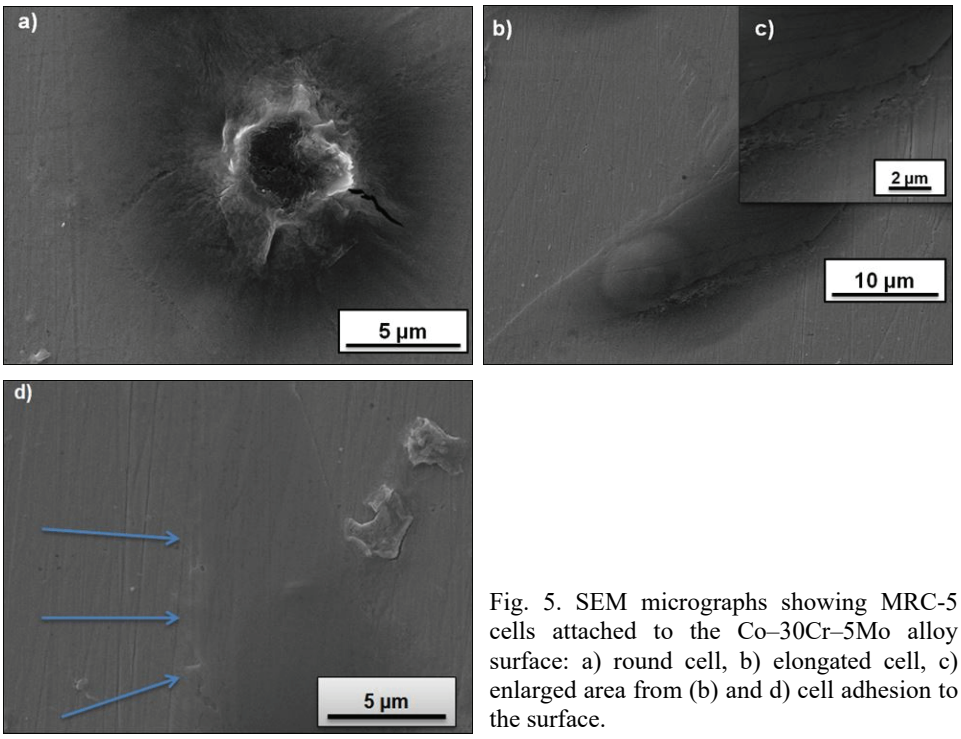

Fig. 5. SEM micrographs showing MRC-5 cells attached to the Co–30Cr–5Mo alloy surface: a) round cell, b) elongated cell, c) enlarged area from (b) and d) cell adhesion to the surface.

CONCLUSIONS

On the grounds of realized and presented research, the following conclusions were reached: 1) The ion release rate of the Co–30Cr–5Mo alloy was small enough, *i.e.*, the quantities of released ions were 100-fold lower than those permitted according to the ISO 22674 standard. 2) The metallic ion release depended on many factors, such as the pH value of artificial saliva and the immersion time. 3) The results of MTT, DET and ADT assays showed that examined

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Co–30Cr–5Mo alloy did not exhibit cytotoxic effect either in contact with human (MRC-5) or animal (L929) fibroblast cells. 4) The human fibroblasts showed excellent adhesion and spreading on the surface of the Co–30Cr–5Mo alloy. Furthermore, the voluminous nature of the cells indicated that the cells were metabolically active and thus the biocompatibility of the Co–30Cr–5Mo alloy was demonstrated.

Based on this *in vitro* biocompatibility examination, it could be concluded that Co–30Cr–5Mo alloy is a biocompatible material that could safely be used in dentistry.

Acknowledgements. The authors gratefully acknowledge support from the Ministry of Education, Science and Technological Development of the Republic of Serbia through the projects III 46010 and ON 174004. The authors would like to express their gratitude to Vesna Kojić, PhD, Oncology Institute of Vojvodina, for her generous help in conducting the cytotoxicity tests and Đorđe Veljović, PhD, University of Belgrade, Faculty of Technology and Metallurgy for the SEM analysis.

ИЗВОД

IN VITRO ПРОЦЕНА БИОКОМПАТИБИЛНОСТИ Co–Cr–Mo ДЕНТАЛНЕ ЛИВЕНЕ ЛЕГУРЕ

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Метални материјали, као што су Co–Cr–Mo легуре, су изложени агресивним условима у усној дупљи која представља идеалну средину за отпуштање металних јона и биоразградњу. Јони отпуштени из денталних материјала могу да изазову локалне и/или системске штетне ефекте у људском организму. Због тога се захтева да дентални материјали поседују одговарајућа механичка, физичка, хемијска и биолошка својства. Биокомпатибилност металних материјала је веома битна за денталну примену. Према томе, циљ рада је био да се одреди отпуштање јона и цитотоксичност Co–30Cr–5Mo ливене легуре, као почетна фаза процене биокомпатибилности. Одређивање вијабилности људских (MRC-5) и животињских (L929) ћелија фибробласта је спроведено применом три теста: колориметријског MTT теста, теста губљења боје (DET) и агар дифузионог теста (ADT). Осим тога, морфологија и раст ћелија су анализирани коришћењем скенирајуће електронске микроскопије (SEM). Добијени резултати указују на то да Co–30Cr–5Mo легура не отпушта штетне елементе у високим концентрацијама које би могле да проузрокују штетне ефекте на људским и животињским фибробластима под датим експерименталним условима. Осим тога, ћелије фибробласта показују веома добру адхезију на површини Co–30Cr–5Mo легуре. Према томе, може се закључити да је Co–30Cr–5Mo легура биокомпатибилни материјал који се безбедно може користити у стоматологији.

(Примљено 5. маја, ревидирано 13. августа, прихваћено 26. августа 2015)

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