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THE EFFECT OF REPEATED CASTING ON THE BIOCOMPATIBILITY OF A DENTAL GOLD ALLOY

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Although highly noble Au alloys have been considered as biocompatible dental materials, little is known about whether recasting affects their biocompatibility. The aim of this work was to study the effect of repeated casting procedures on the microstructure and biocompatibilty of a noble Au dental alloy. The prepared samples of one- four- and eight times melted/cast Dentor S were used to study primary cutaneous irritation and sensibilization on experimental animals. The extract of the alloy prepared by conditioning the Dentor S samples in a cell culture medium was used to study the cytotoxicity on L929 cells. The elemental composition and surface changes of the alloy were examined by Energy Dispersive X-ray (EDX) analysis and optical microscopy, respectively. Our results showed that repeated casting decreased the biocompatibility of Dentor S, manifested as decreased metabolic and proliferative activities of L929 cells and that the effect correlated with the number of melting/casting procedures. However, neither sample of Dentor S caused irritation and sensibilization on experimental animals. EDX showed that recasting slightly incresed the content of Au and Cu and slightly decreased the content of Pt, Pd and Zn. These changes correlated with the observations by optical microscopy. In conclusion, our results show that repeated casting of noble Au alloy changes its microstructure and decreases the biocompatibilty, suggesting that this procedure should be avoided in dental practice.

Key words: biocompatibility, highly noble dental alloy, microstructure

INTRODUCTION

Dental alloys are widely used as constructive materials in the fabrication of dental restorations. Dental alloys are multi-component alloys which consist of solid solutions of metals and non-metals. With the process of alloying, the

required properties of metal can be improved and harmful ones diminished. An alloy is composed of a basic metal and alloying elements, which can be either metals or non-metals (Knosp, 2003).

Among numerous dental alloys, those which are of a special significance are the alloys with the prefix "precious". Precious dental alloys are crystalline materials. Atoms, i.e. elements which form a crystal lattice, are periodically arranged spatially in a regular fashion. Such an order extends throughout the areas, which are very large compared with the inter-atomic distances. The arrangement of atoms in a crystal lattice gives an ideal crystal structure according to the principles of geometric crystallography, which is the most stable structure in all technical materials from the thermodynamic viewpoint. It is true for all materials and, therefore, for precious dental alloys as well, that as solids up to 0 K they are most stable thermodynamically when they are in a crystal form. In special conditions precious dental alloys may harden into an amorphous material, but only if the transition from the melted into a hardened state is fast enough so that atoms cannot be arranged in the thermodynamically most stable position (amorphous metals). Under real speeds of hardening and cooling, which are achieved in dental technical laboratories, a real crystal structure is achieved, which may deviate from an ideal structure. These deviations from the ideal structure are referred to in literature as crystal lattice defects (Knosp, 2003). In real conditions, fabricated precious dental alloys have numerous errors in their basic structure: vacant positions in the lattice, other atoms in positions in the lattice, other or its own atoms lodged in the inter-lattice space, line errors, dislocations, boundaries between grains or phases and other structural imperfections (Fig. 1) (Anžel, 2007).

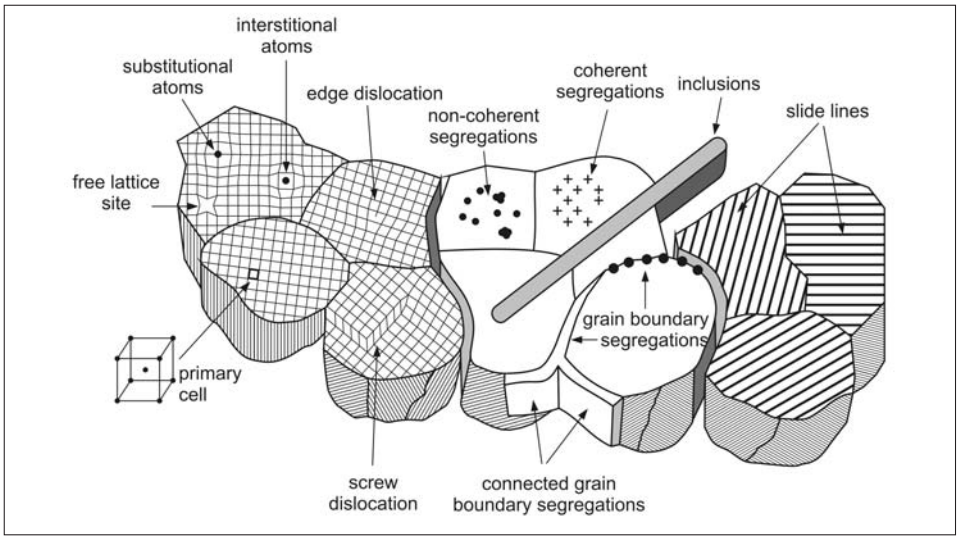

Figure 1. Possible defects in a dental alloy basic structure caused by repeated melting and casting

Over the past 20 years the price of gold has risen significantly in the world market. On one hand, the high price of gold led to a wider use in dental prosthetics of the precious alloys with a lower gold content, as well as the basic alloys. On the other hand, once melted and cast, precious dental alloys are being re-melted and recast. It is often impossible to determine the number of meltings and castings because the weight of new alloy that is added cannot be determined. A dental alloy repeatedly melted and cast is likely to change its chemical composition. Some studies showed that the recasting also changed the biocompatibility of dental alloys due to the lower corrosion resistance of microalloying elements (Rudolf, 2008; Čairović, 2008). Such studies for noble Au alloys are very scarce. Therefore, the objective of this work was to test the effect of repeated casting of a noble Au dental alloy on the cytotoxicity *in vitro*, primary cutaneous irritation and sensibilization on rabbits and guinea pigs, respectively depending on the number of meltings and castings. In addition, these biocompatibility parameters were compared with the changes in elemental composition and microstructure of the alloy in the sample's surface (ISO 1993-10, 1995).

MATERIALS AND METHODS

Preparation of the Au alloy

The dental Au alloy which was used in this research was an alloy widely used in dental practice: Dentor S, Zlatarna Celje (Slovenia). The chemical composition of the alloy is given in Table 1.

Table 1. EDX analysis* of a repeatedly melted and cast Au dental alloy (Dentor S)

No. of alloy meltings & castings	Au m%	Pt m%	Ag m%	Pd m%	Zn m%	Cu m%	No. of analyses
Single melting and casting	75.23	6.57	9.70	1.72	0.96	5.82	12
Four meltings and castings	76.71	5.78	9.35	1.15	0.97	6.04	12
Eight meltings and castings	76.98	5.18	9.70	1.05	0.94	6.14	12

*EDX analysis – energy dispersive X-ray analysis

Samples of the Dentor S dental alloy were obtained after a varied number of repeated casting processes. Test samples of the alloy with the dimensions of 5 mm × 5 mm and a thickness of 2 mm, and 10 mm × 10 mm with a thickness of 1 mm were cast in the induction apparatus in the Dental Laboratory of the Clinic for Dental Prosthetics at the Faculty of Stomatology of Belgrade University. The number of tested alloy samples was 6.

The tested dental alloy samples were as follows: Au alloy which was melted once in the induction apparatus and cast in the conditions of the dental-technical

laboratory; Au alloy which was melted four times in the induction apparatus and cast in the conditions of the dental-technical laboratory; Au alloy which was melted eight times and cast. The samples were sterilized by autoclaving.

Preparation of the Au alloy extract

The extraction procedure included the cultivation of larger disks of Dentor S in 35 mm-diameter Petri dishes with 3 mL of complete culture medium composed of RPMI-1640 medium (Sigma, Munich, Germany) with the addition of 10% fetal calf serum (FCS) (ICN – Costa Mesa) and antibiotics (Galenika, Zemun, Serbia), including gentamycin (10 μg/mL), penicillin (100 units/mL) and streptomycin (125 μg/mL) for 3 days (relative surface area 1.88 cm^2/mL medium) in an incubator (Flow, Irvine, Scotland) with 5% CO_2 at 37°C. After that, media were collected and kept at +4°C. The same procedure was used for the conditioning of a control glass slide of the same size. An additional control medium was kept in the incubator without any tested material.

Biocompatibility testings in vitro

The cytotoxicity of the extracts (conditioning media) was tested using a standard method for measurement of mitochondrial succinic dehydrogenase (SDH) activity in L929 mouse fibroblasts. The cells were cultivated in 96-well plates (ICN) (0.4×10^4 cells/well) with Dentor S conditioning medium, glass conditioning medium or control conditioning medium (6-plikate cultures) in a volume of 200 μL of medium. After 3 days the medium was carefully removed and the wells were filled with 100 μL of 3-[4,5 dimethyl-thiazol – 2 lyl]-2,5 diphenyl tetrazolium bromide (MTT) (Sigma, Münich, Germany) (1 mg/mL) dissolved in complete RPMI medium. Wells with 100 μl of MTT solution served as blank controls. After a 3-hour incubation period (37°C, 5% CO_2), 100 μL / well of 10% sodium – dodecyl sulphate (SDS) – 0.1N HCl (Serva, Heidelberg, Germany) was added to solubilize intracellularly stored formazan. The plates were incubated overnight at room temperature. The optical density of the colour was then measured at 570 nm in a spectrophotometer (Behring ELISA Processor II, Ingelberg, Germany). The results are expressed as the percentage of optical density (metabolic activity) compared to the control (control conditioning medium), defined as 100%.

The viability of the L929 cells was determined after their removal from plastic (Trypsin 0.25%/0.02% EDTA) by using Tripan-blue staining.

The proliferative activity of L929 cells was studied by using a ^{3}H-thymidine incorporation assay. L929 cells (0.3×10^4/well) were plated in 96-well plates and incubated with control or experimental conditioning media for 48 hours. After the addition of ^{3}H-thymidine (for the last 8 hours of cultivation), the cells were detached from the wells by treatement with 0.25% trypsin for 10 min and then harvested. Radioactivity was determined by using a scintilation counter (Beckman). The results were expressed as count per minute (cpm). All experiments were performed in 6-plikates. The values of cpm in the control conditioning medium were defined as 100%.

Test of primary cutaneous irritation

The test of primary cutaneous irritation was performed on experimental animals (albino rabbits of both sexes) bred at the Farm for laboratory animals, Military Medical Academy (MMA), in accordance with the ISO standard 10993-10: 1995 and standards referring to the code of ethics in the treatment of experimental animals (ISO 10993-2: 1992). The age of the animals before starting the experiment was 3 months and the weight was 2.5 kg. Each analysed sample involved three animals. Twenty-four hours before the application of the test preparation, the rabbits were carefully shaved in the back, dorsolateral part of the body over an area of 14 cm × 14 cm.

Test preparations of Dentor S with the diameter of 5 mm were applied once directly onto the shaved skin of each of 3 rabbits onto both the lateral fields cranially, which makes a total of 6 experimental positions for each of 3 preparations. After application the preparations were covered with Senzifix plaster, size 5 cm × 5 cm. Lower positions (6 areas) of each animal served as controls, covered only with the Senzifix plaster (5 cm × 5cm).

All areas were additionally fixed with two parallel Senzifix strips. The tested samples remained in contact with the skin for 4 hours and after that they were removed.

Observations of cutaneous lesions were done 1, 24, 48 and 72 hours after removal of the test preparations. Cutaneous changes (erythema and oedema) on the skin were assessed for each specified period and they were marked according to the Draize numerical scale, (Table 2), (Hayes, 1997; Hugerr *et al*., 2000). The degree of erythema was marked as follows: 0 = without signs of erythema; 1 = mild erythema; 2 = well-defined erythema; 3 = prominent erythema; 4 = severe erythema (deep lesions, crusts, burns, necroses).

Table 2. Effect of repeated casting of Dentor S on primary cutaneous irritation and sensibilization on experimental animals

No. of alloy meltings & castings	PII	IS	Classification of test preparation
Single melting and casting	0	0.026	non-irritating / non-sensibilizing
Four meltings and castings	0	0.033	non-irritating / non-sensibilizing
Eight meltings and castings	0	0.013	non-irritating / non-sensibilizing

PII = Primary cutaneous irritation index; IS = Sensibilization index

The degree of oedema was marked as follows: 0 = without oedema; 1 = very mild oedema; 2 = mild oedema (well defined edges); 3 = moderate oedema (about 1 mm thickness); 4 = severe oedema (thickness exceeding 1 mm, spreading outside the application area).

The irritation score (IS) was obtained by dividing the total sum of the numerical values of oedema and erythema for all three experimental animals by the number of readings. The number of readings referred to the time points (24, 48

and 72 hours) after removal of the preparations (IS = number of readings/oedema + erythema).

Numerical values obtained for oedema and erythema 1 hour after removal of the preparations were not included in the calculation. A primary cutaneous irritation index (PII) was calculated by dividing the value of the irritation score (IS) by the number of application points (PII = irritation score/number of application).

Based on the index of primary cutaneous irritation the preparation was classified as: non-irritating (PII = 0.0 – 0.4); mildly irritating (PII = 0.5 – 1.9); irritating (PII = 2.0 – 4.9); severely irritating (PII = 5.0 – 8.0).

Sensibilization test

The sensibilization test was performed on albino guinea pigs of both sexes, bred at the Farm for Experimental Animals, MMA, by applying the Buehler's method in accordance with the ISO standard 10993-10:1995. This study was also done in accordance with the ethics in the treatment of experimental animals (ISO 10993-2: 1992). The age of animals before starting the experiment was three months and after the experiment it was five months. The body weight of the animals was 300-500 gr. Each test involved 10 animals (30 in total). The same control group, consisting of 5 animals, was used.

Twenty-four hours before application of the test preparation, the guinea pigs were carefully shaved in the front dorsolateral part of the body over an area of 8 cm × 5 cm.

Application I: Test preparations of dental alloys were applied onto the shaved skin (on an area of 2.5 cm × 2.5 cm) of each of 10 guinea pigs (per one preparation) dorsolaterally (cranially, closer to the scapula). After application, the test preparations were covered with Senzifix 5 cm × 5 cm plaster. As control subjects were used, areas of the same size (2.5 cm × 2.5 cm) on 5 guinea pigs, onto which sterile gauze was placed, damped with 0.2 mL of the NaCl isotonic solution and additionally covered with the Senzifix plaster. The tested and controlled samples remained in contact with the skin for 6 hours and after that they were removed.

Application II: Seven days after the first application of the test preparation, the same procedure was repeated on the same experimental animals.

Application III: Seven days after the second application of the test preparation, the same procedure was repeated on the same experimental animals.

Application IV: Seven days after the third application of the test preparation, the same procedure was repeated on the same experimental animals.

Application V: After a 14-day interval following Application IV, the test preparations were placed on previously shaved skin (8 cm × 5 cm), on its untreated area, in the caudal dorsolateral part of the same experimental animals. The tested preparation remained in contact with the skin for 4 hours and after that it was removed.

The treatment of the control group was done at the same intervals as for the experimental group.

Observations of cutaneous changes were done immediately after removal of the test preparations, as well as 24 and 48 hours after each of the five applications. Cutaneous changes on the skin (erythema and oedema) were assessed for each specified period using the criteria given for the irritation test.

The sensibilization score was obtained by dividing the total sum of oedema and erythema numerical values for all ten experimental animals by the number of readings. The number of readings referred to the time points immediately after removal of the preparations, i.e. 24 and 48 hours after removal of the preparations for four successive applications, and separately for the fifth application. The sensibilization score was calculated for the control and experimental groups. (Sensibilization score = (oedema+erythema) / number of readings).

The index of sensibilization was calculated by dividing the sensibilization score value by the number of animals (Index of sensibilization = Sensibilization score / number of experimental animals). The classification of test samples was as follows: non-sensibilizing (IS = 0.0 – 0.3); mildly sensibilizing (IS = 0.4 – 1.5); sensibilizing (IS = 1.6 – 3.0); very sensibilizing (IS = 3.1 – 6.0).

Optical and scanning electron microscopy (SEM)

Microstructural characterisation of all the samples after melting/casting processes and before biocompatibility testing was carried out by optical microscopy (OM-Nikon Epiphot 300 with a system for quantitative analysis) and scanning electron microscopy (SEM-Sirion 400 NC), in addition to energy dispersive X-ray (EDX) analyses (Oxford INCA 350). Concentrations of alloying elements in the analysis phase were performed by at least 12 EDX measurements at different positions on the surfaces of grains. The specimens for optical microscopy were prepared with standard metallographic methods (brushing and polishing) and etched in a mixture of hydrochloric and nitric acids.

RESULTS

The effect of repeated casting on the biocompatibility of Au dental alloy in vitro

The first aim of this work was to study the effect of recasting on the biocomaptibility of Dentor S *in vitro* using the L929 line as target cells. As presented in Materials and Methods, we used an indirect approach by studying the effect of Dentor S conditioning medium. The conditioning medium of once melted/cast Dentor S did not significantly change the metabolic activity of L929 cells, compared to control conditioning media (medium alone or glass conditioning medium). However, recasting of Dentor S caused a statistically significant decrease of the metabolic activity of L929 cells and this effect correlated with the number of melting/casting procedures (Fig. 2A).

The finding in the MTT test was not related to the decrease of cell viability (Fig. 2B), but was a consequence of inhibited proliferation of L929 cells (Fig. 2C).

Effect of repeated casting on the biocompatibility of Dentor S *in vitro*. The biocompatibility of Dentor S was studied by an indirect approach (testing of conditioning medium) on L929 cells as described in Materials and methods. A)

MTT test; B) Viability test; C) Proliferation test. Values are given as mean ± SD (n = 6). *=p<0.05; ***=p<0.005 compared to corresponding controls (glass conditioning medium).

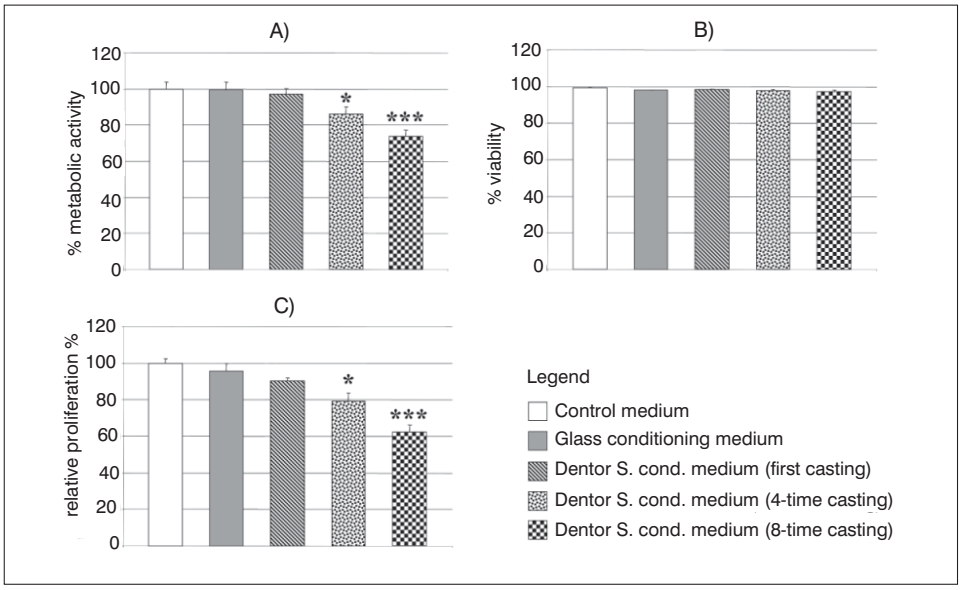

Figure 2. The effect of repeated casting on the biocompatibility of Au dental alloy *in vivo*

We studied next whether recasting procedures of Dentor S can affect its biocompatibility *in vivo*, using irritation and sensibilization tests on laboratory animals. The results presented in Table 1 show that neither sample of Dentor S caused irritation of rabbit skin. The sensibilization tests on guinea pigs showed that some animals in each group had mild signs of erythema. However, according to the sensibilization index (IS) neither cast nor recast Dentor S were marked as sensibilizing alloys (Table 1, Fig. 3).

Figure 3. Photographs of the guinea pig skin following application of Dentor S. A) Application of Dentor S; B) Signs of mild erythema; C) Without erythema and oedema

Microstructural alterations of repeatedly cast Au alloy

Finally, we examined the chemical composition and surface changes of cast and recast Dentor S in order to find the relationship between the biocompatibility and microstructures of the alloy. The results given in Table 2 show that recasting

was followed by a slight decrease in the content of Zn (from 0.96% to 0.94%), Pd (from 1.72% do 1.05%) and Pt (from 6.57% to 5.18%). In contrast, an increase in the content of Au (from 75.23% to 76.98%) and Cu (from 5.82% to 6.14%) occurred. Such changes detected by EDX are in correlation with the changes observed by optical microscopy (Fig. 4). The general features of all optical microstructures are the same, but some changes can be seen. The grain sizes are in the range of 70 μm in all microstructures. The microstructure of the dental alloy which was melted/cast 1× contains two different phases: the yellow bright-coloured base matrix –and the dark coloured minor phase – Fig 4A. Furthermore, the microstructure of the dental alloy which was melted/cast 4× (Fig. 4B) discovered that the minor dark phase became distinctive and more visible, which consequently presents a higher surface fraction degree. Finally, in the microstructure in which the dental alloy was melted/cast 8× the surface fraction of the minor phase grew by up to about 50%.

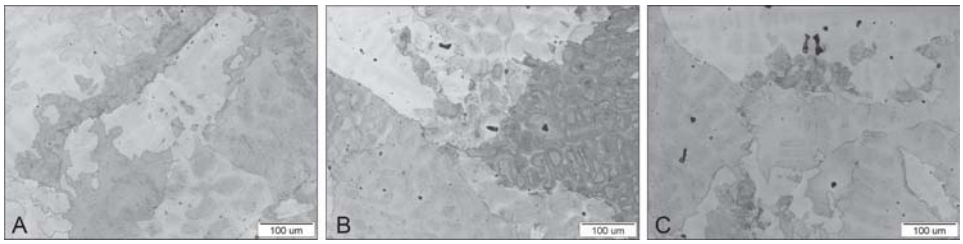

Figure 4. Microstructure of a noble gold alloy (Dentor S) after one melting and casting (A), four meltings and castings (B) and eight meltings and castings (C)

DISCUSSION

When evaluating the dental Au alloys it is important to determine their physical and mechanical properties, but it is also necessary to assess the behaviour of the alloys in the conditions of the oral cavity with regard to their biocompatibility, because these characteristics are often in contrast to each other (Rudolf *et al.*, 2008; Wataha, 1999). During fabrication of dental Au alloys they are thermally treated and enriched with other elements which improve their mechanical properties, but, at the same time, the alloys become less homogenous, which results in their reduced biocompatibility. Of all the criteria which serve to assess the quality of dental alloys, biocompatibility is certainly the most important one, as it combines requirements for the non-toxicity of the ions of dental materials in the human body and for the microstructural, thermodynamical and electrochemical stability, (Namikoshi *et al.*, 1990; Lazić *et al.*, 2008; Geurtsen, 2002; Bayne, 2005; Wataha, 2000).

Our results show that the extract of one time melted/cast Dentor S did not alter the viability, metabolic functions and growth of L929 cells *in vitro*. However, the repeated casting, although it did not change cell viability, decreased the metabolic activity of L929 cells and the process was associated with inhibited

proliferation of the cells. These results are generally in agreement with those published by Al-Hiayasat *et al.*, (Al-Hiaysat *et al.*, 2003) who showed that recasting lowered the corrosion resistance of both basic and noble alloys, manifested as a higher release of microalloying elements. Although we did not study the release of metal ions in culture medium, it can be postulated that Cu, Zn and probably Ag are those elements responsible for the adverse effect on L929 cells (Wataha *et al.*, 1999). Since we did not observe signs of direct cytotoxicity, the inhibition of the proliferative activity of L929 cells can be seen at the sub-toxic concentrations of the released ions (ISO 1993-10, 1995).

Literature data regarding the effect of recasting on changes in elemental composition and microstructure of high noble Au alloys is very scarce. Our results clearly show the presence of such changes, which could explain a decrease in the biocompatibility of recast Dentor S. If we take into consideration that melting/ casting processes normally influence changes to the chemical composition, we can conclude that such changes cause different microstructure formation and also final properties (mechanical, corrosion resistance, etc.).

For explanation of the cytotoxicity of metal ions it is important that their concentration in conditioning fluids reach the levels to cause a detectable adverse effect. The concentrations of released metal ions depends on the corrosion properties of an alloy in an immersion solution, but also on the ratio of surface area of a specimen to the volume of solution and the duration of conditioning. Most authors investigating the corrosion of dental alloys follow the recommendations made by ISO (1997), (surface area-to volume ratio: 0.5-6.0 cm^2/mL; period of conditioning: 3-7 days; immersion solutions: distilled water, acidified artificial saliva or culture media). Our conditioning conditions were: surface area-to volume ratio: 1.88 cm^2/mL; period of conditioning: 3 days. As can be seen, the range of these parameters is rather broad, and because of that it is difficult to make exact comparisons of the results obtained in different laboratories.

Al-Hyasat *et al.* (2003), showed that the release of Zn (167±6.5 ppb) from Bioherador N, a high noble Au-Pt alloy, composed of 86.2 wt.%Au, 11.2 wt.%Pt and 1.5 wt.%Zn in distilled water (0.5 cm^2/mL) was still insufficient after 7 days to cause a significant biological effect. However, the tested concentration was only 10% of the aqueous alloy extract diluted in DMEM medium with 5% FCS.). In comparison with our results, Schmaltz *et al*. (1997), found similar behaviour of two high noble Au alloys regarding their biocompatibility in vitro, although the conditioning conditions were different (0.8 cm^2/mL of Eagle basal culture medium with 5% FCS ; period of conditioning: 7 days).

An important observation from this study is that recasting of Dentor S did not cause irritation on rabbit skin. Some erythema reactions observed on the skin of guinea pigs during the sensibilization test were not marked as sensibilizing reactions. They are probably caused by the treatment of the animals (shaving, desinfection). The sensibilization reactions were non-expected, because neither of the alloy constituents is declared as a strong sensibilizing agent (Čolić, 2009).

In conclusion, our results show that recasting changes the microstructure of Au dental alloys and decreases their biocompatibility, suggesting that such a procedure should be avoided in dental practice.

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UTICAJ PONAVLJANOG LIVENJA NA BIOKOMPATIBILNOST DENTALNIH LEGURA ZLATA

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SADRŽAJ

Visoko plemenite legure su biokompatibilni materijali, ali je malo poznato da li ponovljena livenja legura utiču na njihovu biokompatibilnost. Cilj ovog rada je bio da se utvrdi efekat ponovljenog topljenja i livenja na mikrostrukturu i biokompatibilnost visoko plemenitih dentalnih legura zlata. Pripremjena je serija uzoraka od jednom, četiri i osam puta topljene i livene Dentor S legure za ispitivanje primarne iritacije kože i senzibilizacije na eksperimentalnim životinjama, kao i *in vitro* ispitivanje citotoksičnosti na L929 ćelijama. Hemijski sastav i promena površine legure ispitivana je EDX analizom i optičkim mikroskopom. Naši rezultati su ukazali da ponavljana livenja smanjuju biokompatibilnost Dentor S legure, koja se manifestuje smanjenjem metaboličke i proliferativne aktivnosti L929 ćelija i da je efekat u korelaciji s brojem topljenja i livenja. Ni jedan uzorak Dentor S legure ne uzrokuje iritaciju i senzibilizaciju eksperimentalnih životinja. EDX analiza dokazuje da ponovna livenja blago povećavaju procenat Au i Cu i blago smanjuju procenat Pt, Pd i Zn. Ove promene su u korelaciji sa zapažanjima na optičkom mikroskopu. Autori zaključuju da ponovljena livenja visoko plemenitih legura zlata menjaju mikrostrukturu i smanjuju biokompatibilnost i preporučuju da se ova procedura izbegava u svakodnevnoj praksi.