

IN VITRO EVALUATION OF BIOCOMPATIBILITY OF TWO
DENTAL ALLOYS ON STEM CELLS FROM APICAL
PAPILLA – A COMPARATIVE STUDY

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Abstract

In the present study we performed a comparative in vitro evaluation of biocompatibility of two nickel-containing dental alloys produced by two different methods – conventional and powder metallurgy. Stem cells from apical papilla (SCAPs) were isolated, cultured and incubated in 6% solution of alloys powders in DMSO for 48 hours and further examined for apoptosis, proliferation and cellular metabolism. Our findings suggest that the alloys produced by the traditional method (Crystalloy N) release more electrically charged products of electrochemical corrosion, resulting in increased ROS production in SCAPs, increased percentage of apoptotic cells, decreased proliferative potential and tend to increase antioxidant enzymatic defence. No allergic pro-inflammatory effect of the metal alloys on stem cell cultures was confirmed.

Key words: dental materials, dental alloys, corrosion, haptens

Introduction. *Biocompatibility* is “the ability of a material to elicit an appropriate biological response in a given application” [1]. *Toxicity* of a material is the ability to damage a biological system by chemical means. It is a dose related potential of a material to cause cell or tissue death [2]. Dental materials must be assessed through several toxicity and biocompatibility steps. ISO 7405 is espe-

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cially developed for devices used in dentistry, and adopted the following sequence of *tests for evaluation of dental materials*, i.e. in vitro tests (cytotoxicity, mutagenicity); in vivo tests (sensitization, implantation tests, mucosal irritation) and usage – clinical tests [3].

All dental alloys must meet bio-tolerance standards, but nonetheless, electrochemical corrosion occurs in the oral cavity. *Corrosion* is a deterioration phenomenon of materials due to their chemical reaction with intolerable environment. In macro aspect, the corrosion of dental metal alloys leads to an increase in galvanic current in the oral cavity. If it exceeds 800 mV we are talking about pathogalvanism [4]. On the other hand, the released corrosive products are considered dental haptens that sensitize the body. At micro level, NiCr restorations release corrosion products (NiO, Cr₂O₃) which may affect the viability and decrease cell proliferation. NiCr restorations take a reducing agent measure and O₂ dissolved in saliva acts as an oxidizer. In the biosystem, Ni tends to be released into electrolyte and is generally found in the form of oxidized Ni (Nickel(II) ion) [5].

Stem cells from dental apical papilla (SCAPs) were first identified and characterized by SONOYAMA et al. [6] in human permanent immature teeth and are described as adherent clonogenic with mesenchymal stem cell features. This makes them extremely suitable for tracking changes in parameters in the present study.

The aim of this study was to perform a comparative evaluation of two Ni-Cr dental alloys on the proliferation, cytotoxicity, apoptosis and metabolic activity of SCAP in a model of an immature tooth root.

Materials and methods. Preparation of alloys specimens. The specimens of Marranium FI and Crystalloy N were ultrasonically cleaned with 95% ethanol, rinsed in deionized water and dried with compressed air without oil or water. Then the samples of the alloys were powdered with diamond mounted cutter. According to the utility model with patent No. 3493/23.01.2020 from Patent office of the Republic of Bulgaria, the dental haptens for patch test containing metal powder are in gel form and in 3 to 7 wt. % concentration [7]. For the needs of the study, a 6% solution of metal powders in DMSO was prepared. The dental haptens were prepared in the laboratory at the Department of Chemistry and Biochemistry – Medical Faculty, Medical University – Sofia.

SCAP culture and passage. SCAPs were isolated from freshly extracted immature teeth, by enzyme digestion method in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase. The cells were cultured with Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37 °C in 5% CO₂. When the cells reached 80% of confluence, they were subcultured, using 0.05% of trypsin. For the purposes of this study, cells from passages 2 to 4 were used. The cells were incubated for 48 h in DMSO solutions of both alloys; cells cultured without alloys solutions in the same condition were considered negative group.

Cell proliferation and cytotoxicity detection. Novus' MTT Cell Proliferation Assay Kit (Catalog # NBP2-54883) was used for quantification of viable cells in proliferation and cytotoxicity assay. The method is based on the conversion of water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) compound to an insoluble formazan product. Viable cells with active metabolism convert MTT into formazan, dead cells lose this ability. Thus colour formation serves as a useful and convenient marker of only the viable cells. The measured absorbance (590 nm) is proportional to the number of viable cells. The assay was performed according to the manufacturer's instructions.

Cell apoptosis detection. The apoptosis in SCAPs was detected by using the Novus' Annexin V Apoptosis Kit [FITC] (Catalog # NBP2-29373-100Tests). The assay works in the following manner: cells that are viable are both Annexin V-FITC and PI negative; cells that are in early apoptosis are Annexin V-FITC positive and PI negative, and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. The assay was performed according to the manufacturer's instructions. A flow cytometric analysis was conducted by Navios EX Flow Cytometer – Beckman Coulter.

Cell metabolic activity. SCAPs culture supernatant concentrations of natural and recombinant human Indoleamine 2,3-dioxygenase (IDO) and HO-1/HMOX1 were measured using Novus' Human Indoleamine 2,3-dioxygenase/IDO DuoSet ELISA kit (DY6030-05) and Human Total HO-1/HMOX1 DuoSet IC ELISA kit (DYC3776-2). The assays employ the quantitative sandwich enzyme immunoassay technique. They were performed according to the manufacturer's instructions.

Data analysis. Data was analyzed with parametric *t*-test for the difference in mean values at 95% significance level, using statistical software SPSS, ver. 17.

Results and discussion. A critical concern regarding the clinical application of dental alloys is their biocompatibility. The release of (Nickel(II) ion) from dental alloys due to continuous corrosion in oral complex environment can induce cytotoxicity for prolonged time [8].

The most widely-used biological systems for in vitro toxicity testing of dental materials are cells in culture. Cytotoxicity is often considered to be the first manifestation of poor biocompatibility, thus leading to cell death in the form of apoptosis or necrosis. Apoptosis is a genetically programmed form of cell death that is procedural and controlled by many molecular signalling pathways, such as intrinsic or extrinsic pathway. The extrinsic pathway is triggered by cell surface receptors, while the intrinsic one involves the mitochondria pathway. Apoptosis which is activated via the intrinsic pathway can be induced by oxidative stress and redox changes [9].

The results on the cytotoxic effect of the investigated alloys on SCAPs (MTT assay) are presented in Fig. 1. Our results indicate that SCAPs treated with the hapten equivalent of Marranium FI alloy are with significantly higher proliferative potential (% of viable cells) if compared with the control ones ($P = 0.05$).

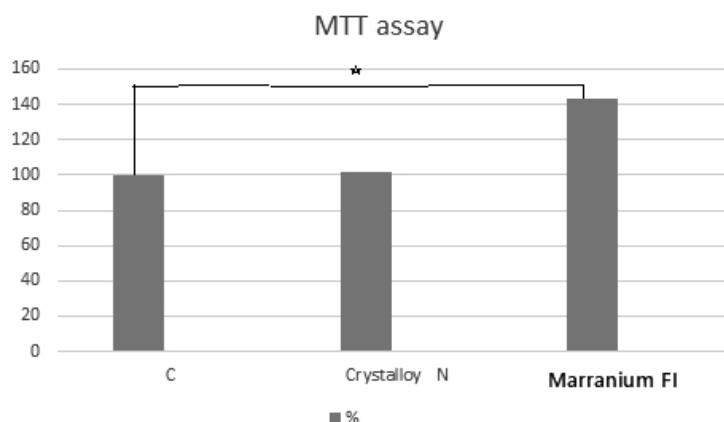


Fig. 1. Viable cells (%) in proliferation and cytotoxicity assay of SCAPs treated with Marranium FI and Crystalloy N

Results regarding the apoptotic effect of dental alloys studied by us on SCAPs are presented in Fig. 2. A significantly higher was the percentage of early apoptotic cells in the group of SCAPs treated with Crystalloy N if compared with the control group ($15.8 \pm 0.6\%$ vs. $7.7 \pm 6.3\%$; $P < 0.050$, $P = 0.041$) (Fig. 2). In addition, significantly lower was the percentage of vital cells in the group of SCAPs treated with Crystalloy N if compared with the control group ($83.7 \pm 0.7\%$ vs. $92 \pm 6.3\%$; $P < 0.050$, $P = 0.04$).

According to our results, treatment of SCAPs with a dental alloy produced by the traditional method, being with higher release of (Nickel(II) ion) (Crystalloy N) results in lower cell proliferative potential and higher percentage of early apoptotic cells. These findings confirm previous statements that the higher (Nickel(II) ion)

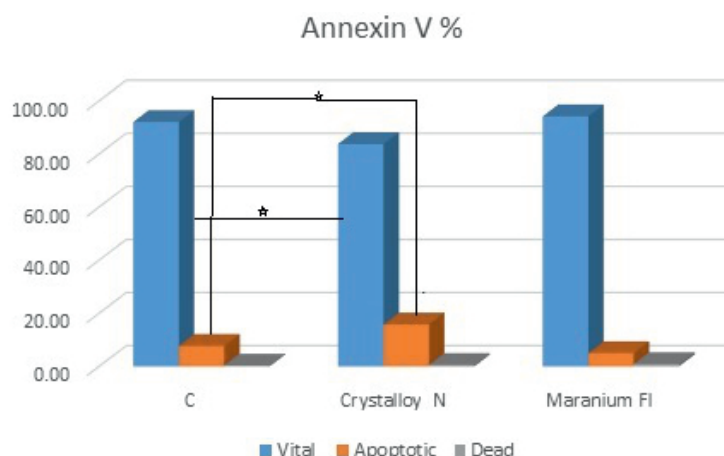


Fig. 2. Apoptotic and viable cells of SCAPs treated with Marranium FI and Crystalloy N

release, the higher the apoptosis. Most probably, this is due to oxidized Ni entering into the cell by means of competing with the essential trace elements (Cu, Zn, Mn, Co, and Fe) required for metabolic enzymes activity. In addition, (Nickel(II) ion) has a high electrode potential allowing it to possess a high affinity for negative charge; it possibly enters into the cell through specific ion channels and stimulates the generation of ROS that causes DNA damage [10].

Nickel, chromium, cobalt, iron, titanium, and molybdenum are all classified as transition metals, which can undergo redox cycling reactions, thus forming reactive oxygen species (ROS) [11]. A recent study shows that chromium(III), iron(III), nickel(II) and molybdenum(III) induce oxidative stress. In the case of chromium(III), nickel(II) and molybdenum(III) the intracellular ROS were dominant [12]. In response to oxidative stress, the organism protects itself by upregulating several enzymes, including heme-oxygenase-1 (HMOX-1).

Data concerning the effect of the alloys on SCAPs culture supernatant HMOX-1 release (ng/ml) are presented in Fig. 3. The addition of Crystalloy N solution to SCAPs culture resulted in higher production of the enzyme (654.7 ± 330.9 ng/ml) if compared with the SCAPs treated with MARRANIUM FI (353.6 ± 330.9 ng/ml) and the control (217.9 ± 39.2 ng/ml), but no significant differences were observed.

In most cell types, IDO is induced at the transcriptional level in response to specific inflammatory stimuli. IFN- γ is the principal IDO inducer in vitro and in vivo [13]. IDO is an antioxidant enzyme because it is a direct scavenger of superoxide radicals [14].

Data on the effect of the alloys on SCAPs culture supernatant IDO release is presented in Fig. 4. Similarly, the addition of Crystalloy N solution to SCAPs culture increased IDO production (0.28 ± 0.5 ng/ml) to a higher extent if compared with the SCAPs treated with Marranium FI (0.16 ± 0.5 ng/ml), again without significant differences.

Nonprecious metal alloys, Marranium FI (Sintal MM, Bulgaria) and Crystalloy N (Sandental, Bulgaria) are a nickel based dental alloy produced by two different methods.

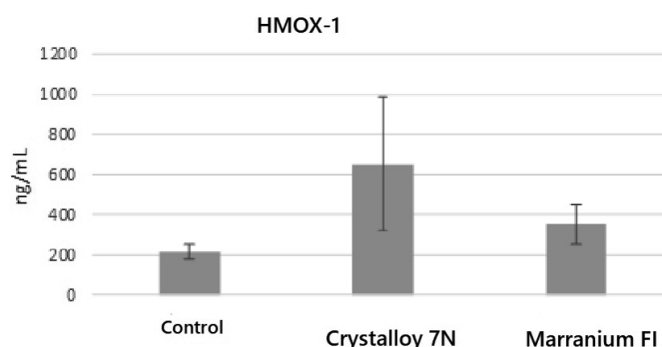


Fig. 3. SCAPs culture supernatant HMOX-1 release

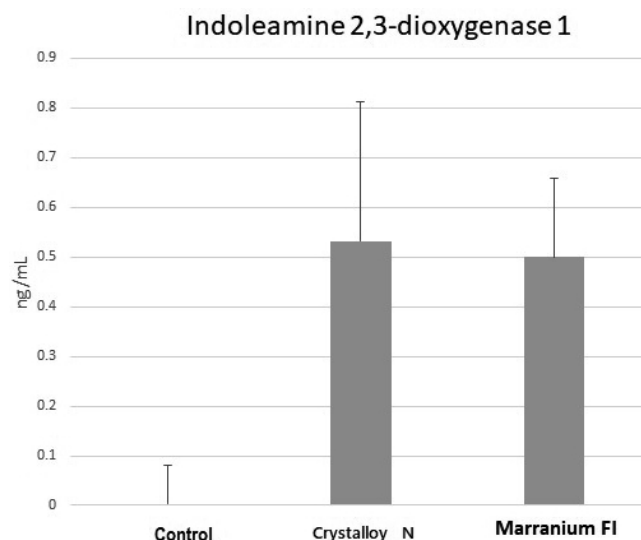


Fig. 4. SCAPs culture supernatant IDO release

The traditional methods of production of dental alloys include consequent procedures of melting of their components, thermal alloying to form solution and molding of ingots. In the traditional thermal alloying process, while some of the components evaporate, others are still not melted. The latter could result in variations in the composition of dental alloys and change in their biological properties, which could be crucial for their biocompatibility. The use of powder metallurgical technique overcomes upper shown difficulties. In that method, homogeneous mixture of fine metal powders is used as starting material and specific methods of cold pressing and sintering are used for shaping and densification of ingots with desirable composition. The advantages of alloys created in this way are that there is an absolutely uniform distribution of the alloy components and a homogeneous melt [5].

Corrosion is an electrochemistry process that involves the transfers of electrons. In order to minimize biological risks, alloys with the highest possible corrosion resistance should be selected. According to recent beliefs, there is no dental alloy that is completely safe. A large number of studies on cytotoxicity, ionic emissions, properties, composition and corrosion resistance of nonprecious dental alloys are available, and the results are not unambiguous.

The cytotoxicity of dental alloys depends on, but is not limited to, the extent of its corrosion behaviour. Corrosion with subsequent metallic ion release is a complex phenomenon that affects all alloys. In case of dental alloys, patients are exposed orally to these constituents, which may lead to adverse reactions, such as gingival swelling, pain, erythema, burning mouth, lichenoid and allergic reactions. Individual ions may have effects on cell viability that are different from metals

interacting within the alloy structure. These adverse reactions may result from local cytotoxic effects and/or immunologic responses [16].

Human mesenchymal stem cells might exert their immunomodulatory function through production of indoleamine 2,3-dioxygenase (IDO) [17]. IDO is a heme enzyme that initiates the oxidative degradation of the essential amino acid ltryptophan along the kynurenine pathway. IDO also represents an important immune control enzyme. Cells expressing IDO are capable to suppress local T cell responses and to promote immune tolerance under various physiological and pathophysiological conditions of medical importance [18]. We could suggest that the increased IDO production from SCAPs treated with Crystalloy N could present a protective response against the oxidative stress induced by the increased release of metal ions from an alloy produced by the tradition method.

HMOX-1 is a member of the oxidoreductase family and catalyses the degradation of heme in carbon monoxide, divalent iron and biliverdin. It is then converted in bilirubin, the most abundant endogenous antioxidant in mammalian tissues, responsible for a number of antioxidant activities due to the action of multiple oxidation factors, including certain heavy metals such as Co and Cr [19].

HMOX-1 is the most consistently found genetic marker induced by skin sensitizers [20].

The results of the expression of IDO and HMOX-1 do not confirm the presence of allergic pro inflammatory effect of the two metal alloys on stem cell cultures.

Conclusion. The results of the scientific experiment give us reason to summarize that the difference in the impact of these two dental alloys on the biocompatibility tests performed by us is due to their structure and method of production. The alloys produced by the traditional method (Crystalloy N) release more electrically charged products of electrochemical corrosion, resulting in increased ROS production in SCAPs, increased percentage of apoptotic cells, decreased proliferative potential and tend to increase antioxidant enzymatic defence. This may be a key mechanism for the impact of other dental materials (haptens) not only on the oral mucosa, but on other organs and systems in the body, which should be studied in more details.

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