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IN VITRO ASSESSMENT OF Ni-Cr AND Co-Cr DENTAL ALLOYS UPON RECASTING: CELLULAR COMPATIBILITY

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The aim of this study was to evaluate the effect of recasting of commercially available Ni-Cr (Wiron 99) and Co-Cr (Dentalit C) dental alloys on physiology of microenvironmental cells. The viability of fibrosarcoma (L929) cells, human embrional fibroblasts (MRC-5) and isolated peripheral blood mononuclear cells (PBMC) was measured by MTT and acidic phosphatase tests. Presence of dying cells was estimated by Annexin/PI staining while the production of intracellular nitric oxide (NO), reactive oxygen (ROS) and nitrogen (RNS) species was determined by DAF-FM diacetate and DHR staining. Recasting of Ni-Cr alloy intensified its cytotoxicity manifested through enhanced free radicals production, induction of cell death and permamently diminished cell proliferation. On the other hand, after initial toxic effect cells adapted to the presence of Co-Cr alloys. Independently of recasting, Co-Cr alloys are more compatible with microenvironment then Ni-Cr alloy. Oppositely, recasting of Ni-Cr alloy promoted its toxicity.

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1. Introduction

Dental casting alloys play a prominent role in the treatment of dental diseases. This role has been changed significantly in the recent years with the improvement of all-ceramic restorations and the development of more durable resin-based composites. However, alloys continue to be used as the principal material for fixed prosthetic restorations and will likely be the principal materials for years to come. No other material has the combination of strength, modulus, wear resistance and biologic compatibility that dental materials must have to in order to survive long term in the mouth as a fixed prosthesis [1]. For example, cobalt-chromium (Co-Cr) and nickel-chromium (Ni-Cr) alloys have become immensely popular in the field of restorative dentistry. The popularity of these

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alloys can be gauged by the varieties of the alloys available in the market [2, 3]. Currently, the majority of commercial dental laboratories are reusing scrap alloy (used once or more times) either alone or in combination with new alloy for fabricating the dental prothesis, considering only the cost factor of the alloy, without any regard to manufacturers instructions or alterations in its properties on reusage [4, 5]. According to clinical implications, recasting of base metal alloys markedly increases their potential cytotoxic effect. It is believed that biologic reactions, in general, are mainly based on the reaction of metal particles eluted from an alloy with biologically active molecules [6]. On the other hand, the microstructural and morphological changes following recasting could negatively influence corrosion resistance, physical and chemical properties that would also affect clinical performance [7]. Since the release of potentially cytotoxic and allergenic elements from dental alloys upon recasting (such as Ni and Co) is well documented in literature [8-10], it is important to perform detailed *in vitro* study considering cellular behavior in the presence of applied alloys, mainly Ni-based and Co-based as those alloys are the most popular in the market [11]. Only a concise *in vitro* study of recasted alloys could possibly provide an insight into the complex nature of cell-material interface regarding both corrosion and surface morphology effect to the vitality of cells seeded onto materials surface. The main purpose of this study is to compare the influence of recasting of commercially available Ni-Cr (Wiron 99) and Co-Cr (Dentalit C) on cellular vitality in close contact with an alloy. Furthermore, this report provides significant information for the future use of recasting methods and possibility to customize interaction at the alloy-tissue interface.

2. Materials and Methods

2.1 Materials

The alloys used in this study were two commercially available base dental alloys: Ni-Cr (Wiron 99) and Co-Cr (Dentalit C). The compositions of these alloys in wt. % as given by the manufacturer are shown in Table 1. Test samples of the alloys with the diameter of 6 mm and the thickness of 1 mm were cast in the induction furnace at the Dental Laboratory of the Clinic for Dental Prosthetics at the Faculty of Stomatology, University of Belgrade. Casting was performed in accordance with the manufacturer's instructions (casting temperature was 1420ºC for Ni-Cr and 1450ºC for Co-Cr alloy). Tested alloys are marked with W (Wiron 99) and D (Dentalit C) with added numerical code which indicates the number of casting. The tested samples of dental alloys were as follows: W1 and D1 alloys were melted once in the induction apparatus and cast in the conditions of the dental-technical laboratory; W4 and D4 alloys which were melted four times in the induction apparatus and cast in the conditions of the dental-technical laboratory; W8 and D8 alloys which were melted eight times and cast. The disc-shaped samples were wet grinded and polished on metallographic polishing table, cleaned with alcohol and distilled water and then air dried. Samples were examined after a various number of repeated casting processes (first, fourth and eighth melting and casting sequence).

Table 1: Investigated dental alloys; composition was provided by manufacturer.

Type of alloy	Alloy	Composition (wt.%)						Other elements
		Ni	Co	Cr	Mo	Nb	C	
Ni-Cr	Wiron 99	65	-	22.5	9.5	1	0.02	Fe, Si, Ce
Co-Cr	Dentalit C		61	30	5.5	1	0.01	Fe, Si, Cu, Mn

2.2 Cell culture and reagents

Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). Annexin V-FITC (AnnV) was from Biotium (Hayward, CA). Dihydrorhodamine-123 and DAF-FM diacetate were from Molecular Probes (Eugene, OR). Murine fibrosarcoma cell line L929 was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Human embryonic fibroblasts (MRC-5) were purchased from ATCC. Cells are routinely maintained in HEPES-buffered RPMI-1640 medium (PAA Laboratories, Pasching, Austria) supplemented with 10% FCS, and antibiotics (culture medium) at 37ºC in a humidified atmosphere with 5% CO_2. For the experiments, cells were detached by standard trypsinization, resuspended in culture medium and seeded at 1×10^4 /well in 96-well plates for MTT determination, 7×10^4 /well for acidic phosphatase and 2.5×10^5 / well in 6-well plate for flow cytometry. Isolation of peripheral blood mononuclear cells (PBMC) was performed in following procedure: heparinized blood was taken from healthy volunteers and centrifuged at 115 g for 10 minutes. Plasma was layered over 4 ml of Histopaque®-1077 Lymphocyte separation medium (Sigma) and centrifuged for 20 min at 800 g. The cells in interface layer were harvested carefully to prevent contamination with platelets, and washed twice in PBS (10 min at 700 g). Finally the cells were resuspended in RPMI 1640 medium containing 10 % FCS. The cells were stained with trypan blue dye to distinguish dead cells (incorporate the dye) from live cells (exclude the dye) and counted by using an improved Türck-Bürcker hemocytometer. Cells were seeded at 1×10^5 /well in 96-well plates for acidic phosphatase determination, and 3×10^5 / well in 6-well plate for flow cytometry.

2.3 MTT assay

MTT test is based on the reduction of MTT into formazan dye by active mitochondria of living cells. At the end of cultivation 50 µl of MTT solution (0.5 mg MTT/ml in culture medium) was added to each well and plates were incubated at 37°C for 1 hour. To dissolve formazan, which amount was proportional to the number of live cells, DMSO was added. Absorbance of the dissolved dye was measured at 540 nm using an automatic microplate reader (LKB 5060-006).

2.4 Acidic phosphatase assay

Cells (7×10^4 for adherent or 1×10^5 cells/well for nonadherent) in 100 µl of culture medium were cultivated in the presence of different dental materials for 24, 48 and 72 h. At the end of treatment, cells were incubated for 2 h at 37°C with substrate for acidic phosphatase (1.1 mg/ml p-nitro-phenyl-phosphate, 0.4 % Triton-X, 0.3% Na-acetate) and reaction was terminated by addition of 50 µl 1.3M NaOH. The absorbance of developed color was readed at 405 nm using an automatic microplate reader (LKB).

2.5 Apoptosis assay by annexin V-FITC/PI staining

Early apoptotic cells characterized by phosphatidylserine exposure to extracellular environment while cell membrane integrity remains intact were detected using an annexinV-FITC/PI staining kit (Biotium, Hayward, CA). Cells were exposed to dental materials for 48 h, trypsinized and stained according to the manufactureur's instructions. Cells were analysed with a FACS Calibur flow cytometer (BD, Heidelberg, Germany) and the experimental data were processed with the aid of Cell Quest Pro software (BD).

2.6 Measurement of intracellular nitric oxide (NO), reactive oxygen (ROS) and nitrogen (RNS) species

The cells were stained with 1 µM dihydrorhodamine-123, 20 min before the exposure to dental materials. After the cultivation period (24 h), cells were washed and resuspended in PBS.

Analysis of ROS and RNS production was done with a flow cytometer and analyzed using CellQuest Pro software. For intracellular NO measurement, cells were incubated 1 h at 37°C with 2 μM NO indicator DAF-FM diacetate (Molecular Probes) in phenol red- and serum-free RPMI 1640. The cells were then washed and incubated for additional 15 min at 37°C in fresh RPMI 1640 for completion of deesterification of the intracellular diacetates. Finally, the cells were resuspended in PBS and analyzed by flow cytometer.

2.7 Statistical analysis

To analyze the significance of the differences between each alloy and corresponding recasts (D4, D8, W4, W8.) performed in triplicate, we used analysis of variance (ANOVA), followed by the Student-Newman- Keuls test. A p value less than 0.05 was considered significant.

3. Results

3.1 Effect of alloys and corresponding recasts on L929 fibrosarcoma viability

Cells were cultivated in the presence of dental alloys for 24, 48 and 72 h and thereafter cell viability was estimated by two tests, MTT and acid phosphatase. As judged by MTT assay (Fig. 1A), inhibition of mitochondrial respiration was observed upon 24 h long exposure of cells to both types of dental alloy samples (Co-Cr, Dentalit C, marked as D1; Ni-Cr, Wiron 99, marked as W1; Fig. 1) as well as their recasted forms (D4-8 and W4-8; numbers indicate number of recasts; Fig. 1, this notation is adopted in all reported results). The effect was sustained during 3 days of cultivation. Figure 1B displays results acquired by acidic phosphatase assay of L929 seeded on dental alloys. The number of viable cells was not significantly changed after 24 h of cultivation in comparison to control cells cultivated on tissue culture plates in medium without dental materials. However, the rate of proliferation and viability of cells within next 48 h was slightly affected by the presence of D1-8 samples while cell viability upon exposure to W1-8 samples considerably decreases depending on the number of recycling (Fig. 1B).

3.2 Effect of alloys and corresponding recasts on fibroblasts and PBMC viability

Acidic phosphatase assay of MRC-5 fibroblasts is presented in Fig. 2A. There was no difference between viability of cells seeded in culture plates (control) without dental implants after 24 h. However, moderately diminished cell viability of MRC-5 fibroblast cells exposed to both Ni-Cr and Co-Cr alloys has been observed after 48 h in culture (D and W alloys on Fig. 2A). After 72 h significant decrease in number of viable MRC-5 fibroblasts in the presence of Ni-Cr (Wiron 99) alloy, in regards to casting cycles (Fig. 2A) was evident. Importantly, cell proliferation rate after 48 h of cultivation (marked as difference in number of cells after 72 vs. 48 h) in the presence of Co-Cr (D1-8) alloys was similar to control indicating that after initial toxic shock, cells adapted to the presence of this material. In contrast, proliferative capacity of cells cultivated on Co-Cr alloys was permanently diminished. Figure 2B shows results obtained for PBMC seeded on dental alloy samples (both Dentalit C and Wiron 99) in time intervals of 24 and 48 h. These cells are low proliferative cells in the absence of external stimuli and often manifest spontaneous apoptosis in prolonged incubation. For that reason, PBMC viability was measured within 48 h and results are presented as percentage of control. There was no significant difference in cell viability during first 24 h of cultivation in the presence of all alloy samples (Fig. 2B). After 48 h, viability of PBMC decreased upon exposure to W8 sample only (Fig. 2B).

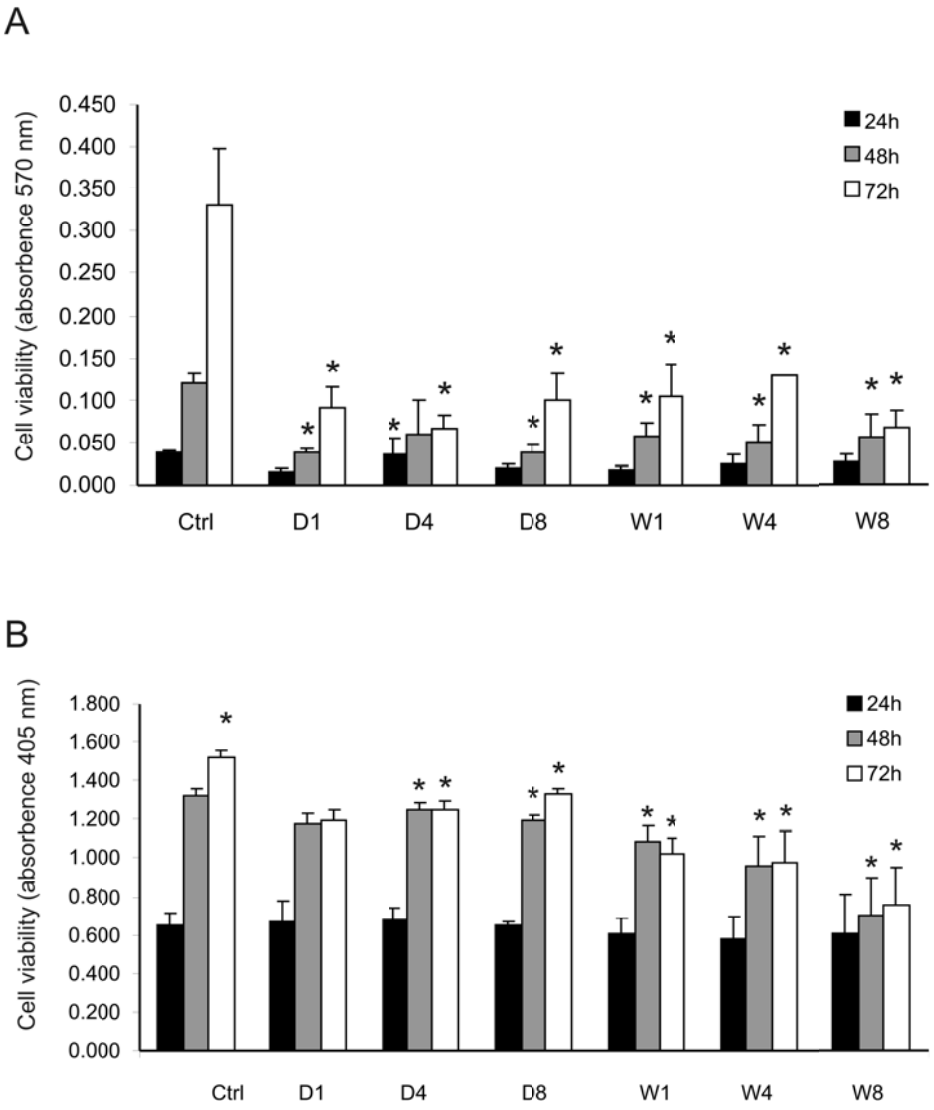

*Fig. 1. The effect of dental materials on L929 fibrosarcoma. L929 cells (7×10^3 cells/well or 3×10^4 cells/well for MTT and acidic phosphatase, respectively) were incubated in the presence of D1-8 and W1-8 samples for indicated time, and then cell viability was determined by MTT (A) and acidic phosphatase (B) assays. The data are presented as mean ± SD from representative of three independent experiments. *$p < 0.05$, refers to untreated cultures.*

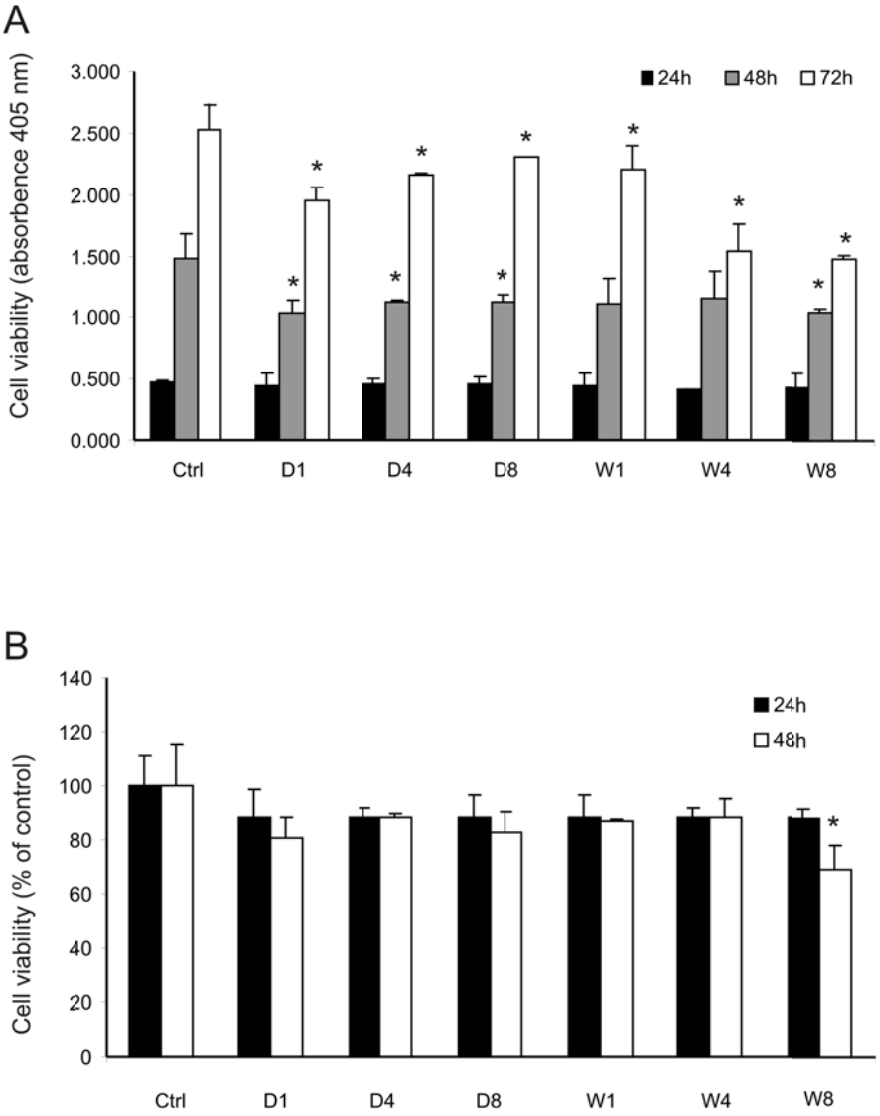

*Fig. 2. The effect of dental materials on MRC-5 fibroblasts and PBMC. (A) Embrional fibroblast cells (3 × 10^4 cells/well) and (B) PBMC (1 × 10^5 cells/well) were incubated in the presence of D1-8 and W1-8 samples for indicated time, and then cell viability was assessed by acidic phosphatase assay. The data are presented as mean ± SD from representative of three independent experiments. *p < 0.05, refers to untreated cultures.*

3.3 Induction of fibroblasts and PBMC cell death in the presence of alloys and corresponding recasts

To investigate the influence of dental material on the cell death, MRC-5 fibroblasts and PBMC were incubated with different samples for 48 h and then the presence of early apoptotic, marked as Ann^+PI^-, and late apoptotic/necrotic cells, marked as Ann^+PI^+, were analyzed by flow cytometry as presented on Fig. 3. Elevated proportion of both early and late apoptotic cells was found in both MRC-5 (Fig. 3A) and PBMC (Fig. 3B) cultures exposed to both Dentalit C (D1-8) and Wiron 99 (W1-8) dental alloys. The induction of apoptosis was more pronounced in Ni-Cr (W1-8) samples and showed tendency of increase in correlation with the number of recasts (Fig. 3A and B).

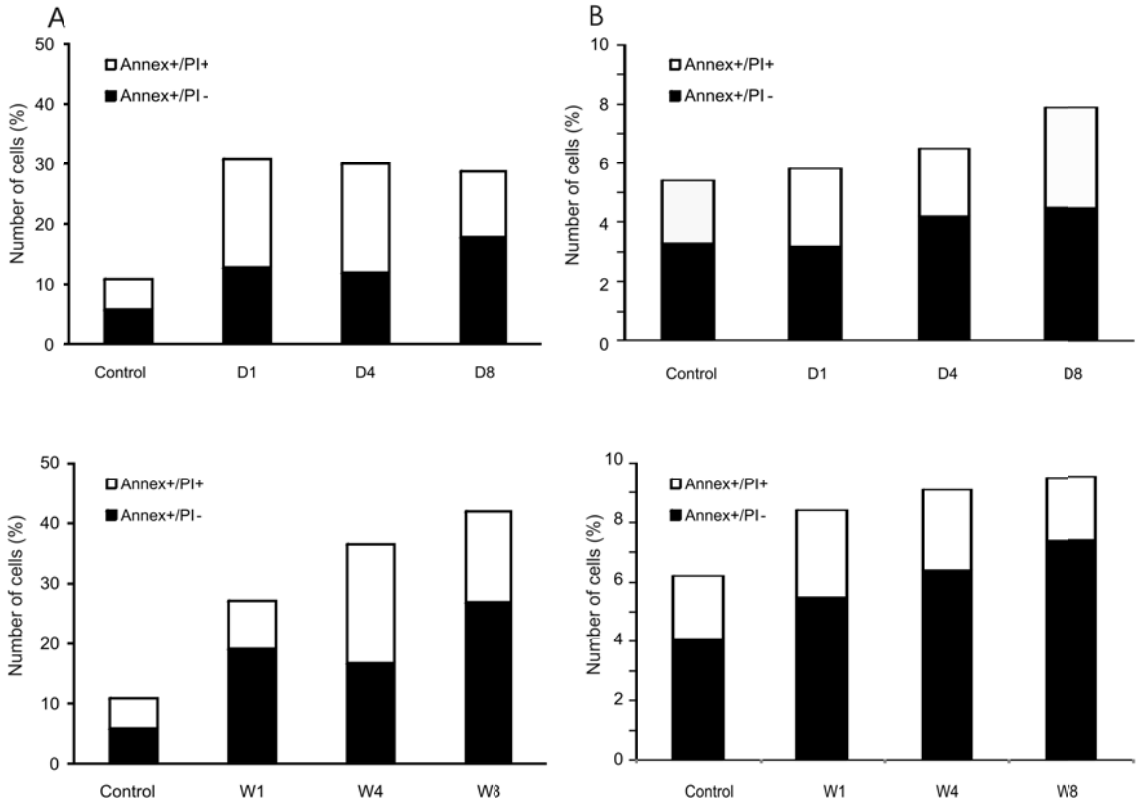

Fig. 3. Dental materials induced apoptotic cell death. (A) MRC-5 fibroblast cells and (B) PBMC were exposed to D1-8 and W1-8 samples and staining by AnnV/PI was performed and evaluated by flow cytometry after 48 h.

3.4 Effect of alloy and corresponding recasts on ROS, RNS and NO production

Production of highly reactive molecules, ROS and RNS that could be mediators of cell death was measured for MRC-5 fibroblasts and PBMC on dental alloys. Data are presented in Fig. 4. Intracellular NO, ROS as well as RNS production determined by specific indicators DAF-FM and DHR, respectively, showed intensified production after only 24 h of exposure to dental alloys, indicating their involvement in initial events triggered by the presence of dental materials. However, level of produced ROS and RNS was remarkably higher in cultures of cells exposed to Ni-Cr (W1-8) samples (Fig. 4A and B). Actually, it is indicative that recasting facilitates production of reactive species with dominant effect observed upon incubation with Ni-Cr (W1-8) samples, highlighting the connection between quantity of ROS/RNS and toxic effects of recasted Wiron 99 Ni-Cr alloy (Fig. 4A and B).

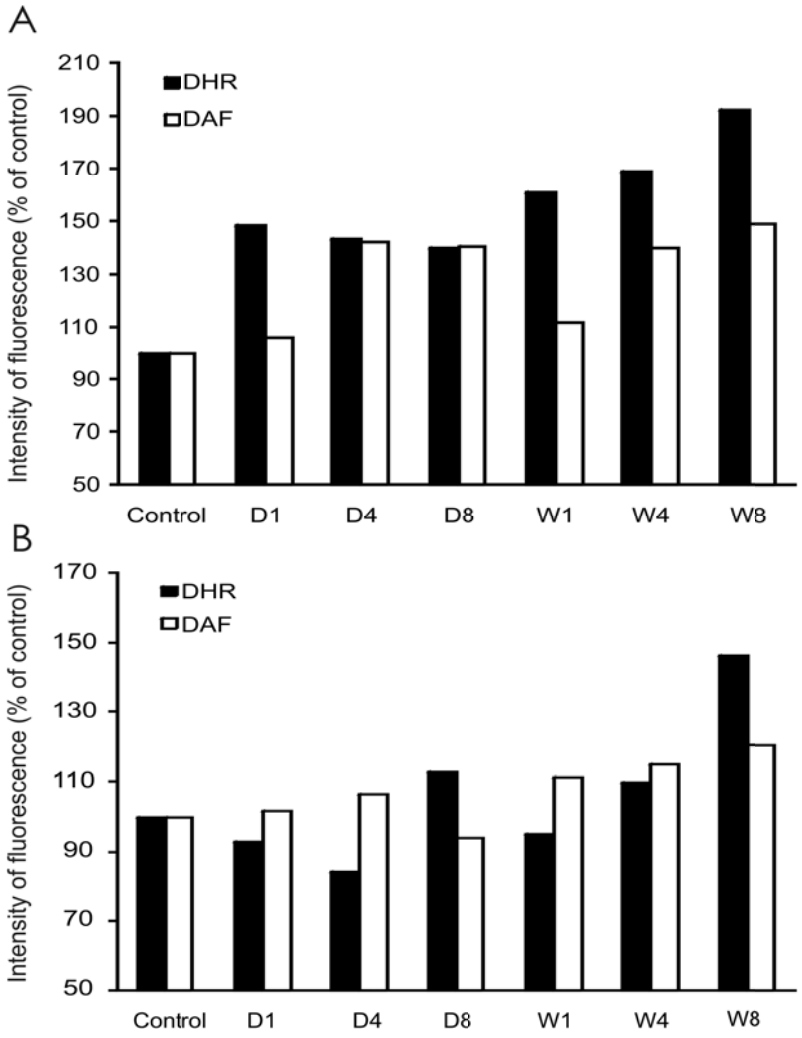

Fig. 4. Dental materials induced NO, ROS and RNS production. (A) MRC-5 fibroblast and (B) PBMC cells were exposed to D1-8 and W1-8 samples and intracellular NO production was determined by DAF-FM indicator and ROS/RNS production by DHR staining. Intensity of fluorescence was analyzed by flow cytometry.

4. Discussion

The biocompatibility assessment of dental materials presents the essential part of screening process for evaluation of their clinical potential. The primary potential of *in vitro* tests is the control of the cell - material interface and the interaction environment. Other important aspect of *in vitro* tests is the ability to assess cellular response with considerable precision [12]. To evaluate the potential toxicity of different dental materials in correlation with the number of recasts, in the present study preliminary experiments were performed on mouse fibrosarcoma cell line L929. This screening cell viability test is recommended by ISO (1997) for the evaluation of biocompatibility of dental materials [13]. Mitochondrial respiration of cells seeded on alloy surfaces was measured by MTT assay and initial drop in cell viability was recorded for all samples in comparison to control. On the other hand, analysis of cell viability according to acidic phosphatase activity was highly inconsistent with data obtained by mitochondrial respiration measurement. In fact, acidic phosphatase assay revealed that decreased mitochondrial respiration did not correlate with the number of viable cells in culture but rather reflected prompt response of the cells to toxic stimuli such as presence of dental material in cultures. Thus, these preliminary experiments showed clearly that mitochondrial respiration is not reliable parameter for cell

viability determination. For that reason, MTT test was excluded from further evaluation of cell viability.

As shown in presented results, a number of recycling of dental alloys influenced the viability of fibrosarcoma cells. Therefore, the effect of dental materials was further assessed on viability of primary fibroblasts (MRC-5) and PBMC, as the most relevant cells present in dental tissue microenvironment. Similarly to fibrosarcoma, moderately diminished cell viability of their primary counterparts MRC-5 exposed to both Ni-Cr (W1-8) and C-Cr (D1-8) alloys has been observed after 48 h. In concordance with this, presence of dying (apoptotic and necrotic) cells was observed in cultures with both dental materials tested. The effect was more pronounced in the presence of Wiron 99 (Ni-Cr) material. The observed phenomenon evidently correlated with number of Ni-Cr (W1-8) recasts. This result is in agreement with the published studies that indicate higher cytotoxicity of Ni-Cr alloys in comparison to Co-Cr and other metal alloys [7].

Oppositely to Ni-Cr (W1-8) alloy, cells survived the contact with Co-Cr (D1-8), rapidly adapted to its presence and retained their proliferative capacity. PBMC, leading cells in immune response to infection or tissue destruction, revealed less sensitivity to the presence of dental material in comparison to other tested cells. Beside this, intensified cytotoxicity of recycled Ni-Cr samples was evident in these cell cultures confirming previous reports that indicate generation of reactive oxygen species (ROS) by Ni ions and induction of oxidative stress, which are the factors responsible for the rapid loss of cellular viability and potential immune response [6, 11]. Taken together, it is obvious that recasting of Ni-Cr (Wiron 99) but not Co-Cr (Dentalit C) material endorsed toxicity of dental Ni-containing alloys [11, 14].

Nickel is also known to have allergenic effect, and the use of Ni-containing materials demonstrated that the risk of nickel sensitization depends upon metal release over time [7, 11]. On the other hand, some studies pointed out higher cytotoxicity of the commercial Co-Cr alloys when compared to other commercial Ni-Cr alloys upon multiple recasting [15]. Furthermore, some authors reported that cobalt is more toxic than nickel [15, 16]. In all cases, the release of ions and subsequent interaction with the tissue environment is a complex phenomenon that depends not just on the composition of the alloy but also strongly depends on the fabrication process, casting technique, surface composition/morphology, multiple-phase consistency and local environment conditions such as pH and concentration of proteins and amino acids [6, 10, 13, 17]. For the reasons above, it is necessary to perform detailed cytotoxicity experiments on available dental alloys in order to understand the cellular behavior in close proximity to the metal surface and to reveal complex processes that occur at the cell - material interface [12, 18].

Further biocompatibility studies will continue to asses fabricated dental alloys and the possibility of systemic toxicity [10]. These findings are important not just for the future of dental alloys but also for manufacturing of a high level nickel-based “shape-memory” wires increasingly used in orthodontics [17]. Although both cobalt-based and nickel-based alloys are most likely to be used by clinicians in many years to come, the results from this study indicate obvious cytotoxicity and strong possibility of immunological response upon alloy recasting, experimental facts that should not be neglected in future practice.

5. Conclusion

The results of the present investigations demonstrate the effect of recasting of commercially available Ni-Cr (Wiron 99) and Co-Cr (Dentalit C) dental alloys on cellular viability of fibrosarcoma (L929) cells, human embrional fibroblasts (MRC-5) and isolated peripheral blood mononuclear cells (PBMC). The number of viable cells was significantly affected upon exposure to Ni-Cr alloy depending on the number of recasts and the cytotoxic effect was the strongest for Ni-Cr alloy after maximum of 8 recasts investigated in this project. However, Co-Cr alloys, both original and recasted counterparts demonstrated better cell compatibility than Ni-Cr alloy with all tested cell types.

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