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Preconditioning of dental alloys: Analysis of fibroblast proliferation and expression of fibronectin and chondroitin sulfate

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The aim of the present study was to analyze the influence of different preconditioning treatments with either bovine serum albumin or cell culture medium of different dental metal alloys on human fibroblast cultures in the presence of such biomaterials as regards both cell proliferation rates and the expression of molecules constituting the extracellular matrix. Human fibroblasts (cell line Flow 2002) were cultured for 72 h in the presence of six single-phase dental casting alloys. The amount of Ag^{+} and Cu^{++} release into cell culture media was measured by atomic absorption spectroscopy. Incorporation of 5-bromodeoxyuridine, to investigate cell cycle, and the expression of fibronectin and chondroitin sulfate glycosaminoglycans, to evaluate cell adhesion, were analyzed with an immunocytochemical approach and related to cytocompatibility of the different substrates. The immunocytochemical analysis were performed by fluorescence microscopy and further analyzed with an image analysis software. Preconditioning treatments for 72 h induced decreasing cytotoxicity of the tested alloys: indeed metal cation concentrations decreased in cell culture media in the presence of preconditioned dental metal alloys. Both cell proliferation rates and ECM-constituting molecule expression resulted higher when tested in the presence of preconditioned dental metal alloys. Therefore, it is reasonable that preconditioning treatments of dental alloys influenced their interactions with fibroblast cultures by increasing their cytocompatibility *in vitro*.

1. Introduction

Biocompatibility and cytotoxicity of dental metal alloys are commonly evaluated by means of *in vitro* tests that analyze the possible adverse side effects resulting from the interaction between cell cultures and the tested metal biomaterials. Previous studies on the cytotoxicity of dental alloys have been focused on both the release of metal ions [1–3] and their interactions with human cultured cells [4], since they have been considered as main biocompatibility parameters. Ions released by dental metal alloys could affect several cellular biological pathways influencing both cell proliferation rates and cell adhesion to substrata by means of different biomolecules constituting the extracellular matrix (ECM) [4–11]. However, *in vitro* investigations could often present controverse clinical relevance if compared to *in vivo* studies on laboratory animals [4, 12–14]. Since metal alloys continuously release ions into biological media, investigations based on long periods (i.e., over 10 months) should be considered more appropriate [15] than short term tests [16, 17]. On the other hand, previous studies on the biocompatibility of

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dental alloys have suggested the utility of short term *in vitro* tests to investigate the possible cytotoxic actions of different dental biomaterials, on the bases that no significant variations of biological parameters were found after extended exposures of biomaterials to biological media [18].

Since a large number of new casting alloys proposed for clinical appllications in restorative dentistry in the latest years, current investigations have been focused on developing new and reliable biocompatibility tests *in vitro* to assess the cytotoxicity and the cytocompatibility of such new biomaterials. In particular, Nelson *et al.* have recently proposed the utility of a preexposure of dental casting alloys for 72 [19] or 168 h [20] to three different biological solutions (i.e., one among 0.8% NaCl solution, complete cell culture medium, 0.8% NaCl solution with 3% bovine serum albumin) before testing their cytotoxicity in cell culture systems. The rationale for this procedure have been based on the possibility of alloy preconditioning treatments to remove more ionizable metal elements from biomaterials, as to reduce alloy cytotoxicity *in vitro*, and to eliminate short-term change providing a corrosion pattern that is more indicative of long-term *in vivo*.

In the assessment of biomaterial biocompatibility, cell viability and cell proliferation rate are two of the most studied biological parameters [15, 21–24] on *in vitro* experimental models with stabilized cell lines [19]. The analysis of the incorporation patterns of a thymidine analogue, such as 5-bromodeoxyuridine (BrdU), into cell nuclei during the S-phase of the cell cycle is a useful technique to study cell proliferation rates [25, 26]. Moreover, it is well known that cell proliferation is strictly correlated to cell capability to adhere to substrata, therefore it seems appropriate to investigate both these biological aspects in relationships to the presence of different dental biomaterials.

In previous studies on cultured fibroblasts on dental biomaterials, we proposed that fibronectin (FN) and chondroitin-sulfate glycosaminoglycans (CS) arrangement in the extracellular matrix (ECM) could be useful tools to evaluate cell adhesion *in vitro* in biomaterial biocompatibility evaluation [21, 23, 27–29].

On these bases, the aim of the present study was to determine the influence of two preconditioning solutions (0.8% NaCl with 3% bovine serum albumin or complete cell culture medium) on the cytotoxicity of six dental metal alloys. Cytotoxicity was assessed *in vitro* by quantifying the concentration of ions released from biomaterials into culture media and by analyzing different biological aspects (i.e., cell proliferation rates and the immunocytochemical expression of FN and CS) of the human fibroblast line Flow 2002. The hypothesis was that alloy pretreatments using the two above mentioned solutions would increase the release of ions from dental alloys and therefore it could change alloy cytotoxicity in comparison to the unconditioned alloys.

2. Materials and methods

2.1. Alloys and preconditioning treatments

Six single-phase dental casting alloys (manufactured by Nobildent srl, Milano, Italy) were previously identified as A, B, C, D, E, F. Subsequently, according to specification #5 of the American Dental Association and to their elemental composition (Table I), the alloys A (Au-Pt alloys), C, D and E (Au-Ag-Cu alloys) were classified as high-noble alloys, the alloys B (Pd-Cu-Ga alloys) and F (Pd-Ag-Cu alloy) were classified as noble alloys.

Metal blocks of each dental alloy (dimensions 1.2 $\times$ 0.7 $\times$ 0.1 cm, total surface area 2.06 cm^2, weight from 4.85 to 7.92 g) were sterilized by exposition for 24 h to an ultraviolet lamp (wave legth 254 nm, UV ray dosage 30,000 μW-sec/cm^2) contained in a laminar flow hood. Specimens were randomly divided and assigned to three different groups of treatment:

– Group 1: alloy preconditioning was performed by using the preconditioning solution 1 (PS1) containing 0.8% NaCl added with 3% (wt/vol) bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO, U.S.A.);
– Group 2: preconditioning was performed by using the preconditioning solution 2 (PS2) constituted by cell culture medium (Minimum Essential Medium, MEM; Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal calf serum (FCS, Sigma, St. Louis, MO, U.S.A.), 100 U/mL penicillin and 100 μg/mL streptomycin sulfate (Sigma, St. Louis, MO, U.S.A.);
– Group 3: metal blocks of the six dental casting alloys did not undergo any preconditioning treatment.

Preconditioning treatments were performed by immersing the metal specimens into 0.5 mL of preconditioning solutions (ratio between the total area of metal blocks and solution volume was 4.12 cm^2/mL) contained in sterile polystyrene cell culture trays and by leaving them there undisturbed for 72 h in a fully humidified air atmosphere containing 5% CO_2 at 37°C.

Finally, the metal specimens were placed on sterile gauze to remove residual preconditioning solution, rinsed briefly in sterile water and at once transferred into cell cultures.

2.2. Cell cultures

Human embryonic lung fibroblasts of the stabilized cell line Flow 2002 (kindly provided by dr. G. Dal Molin, Department of Public Health, University of Trieste, Italy) were cultured in 75 cm^2 flasks in Minimum Essential Medium (MEM, Sigma, St. Louis, Mo, USA) supplemented with 10% fetal calf serum (FCS; Sigma, St. Louis, Mo, USA), 100 U/mL penicillin and 100 μg/mL streptomycin sulfate, at 37°C in a fully humidified air atmosphere containing 5% CO_2.

To assay cell proliferation rates, 10^4 cells were detached from cell culture flasks and spread on sterile coverslips, and then put into 150-mm Petri dishes each containing 5 mL of cell culture medium and left there for 72 h in the presence of one metal alloy specimen without renewing the culture medium. Each cell culture test was performed in duplicate and repeated five times, for a total of ten replications. Eighteen culture specimens were performed: six in the presence of the unconditioned specimens, six in the presence of the PS1 preconditioned specimens and six in the presence of the PS2 preconditioned specimens. Further six cell cultures were performed in the same experimental conditions of

TABLE I Metal composition of six dental metal alloys

Alloys	*Nobility%	Au	Pt	Pd	Ag	Cu	Sn	In	Ir	Ga	Zn	Rh	%	American dental association classification
Au-Pt (A)	98	85	9	3.1	<1.5	<1.5	<1.5	<1.5	<1.5	<0.5	<0.5	<0.5	wt%	High noble
	85.3	72.8	7.7	4.8	2.1	3.8	2	2.1	1.1	1.1	1.1	0.6	at%	
Pd-Cu-Ga (B)	82	2	<1.5	79.5	<0.5	8.5	2.5	<1.5	<1.5	7			wt%	Noble
	73.1	0.9	0.6	71.6	0.3	12.7	2	1.2	0.6	9.5			at%	
Au-Ag-Cu (C)	78	75.5	1.7	<1.5	13.5	>5	<0.5	<0.5	<1.5	<0.5	<1.5		wt%	High noble
	62	58.7	1.2	2.1	19.1	11.9	0.6	0.6	1	1	3.3		at%	
Au-Ag-Cu (D)	65	59	2.2	3.3	19	>5	<0.5	<0.5	<1.5	<0.5	<1.5		wt%	High noble
	53.2	46.7	1.7	4.8	27.5	12.2	0.6	0.6	1	1	3.4		at%	
Au-Ag-Cu (E)	55	50	<1.5	4	29.5	>5	<0.5	<0.5	<1.5	<0.5	<1.5		wt%	High noble
	42.8	36.5	1	5.3	39.4	11.2	0.5	0.5	1	0.7	3.1		at%	
Pd-Ag-Cu (F)	30	1	<1.5	28.5	59	>5	<0.5	<0.5	<1.5	<0.5	<1.5		wt%	Noble
	29.3	0.5	0.7	28.1	57.6	8.2	0.4	0.4	0.7	0.7	2.3		at%	

at% = atomic weight percentage; wt% = weight percentage.
*Sum of weight percentages for Au, Pd and P.
High noble = Au ≥ 40%, Au + Pd + Pt ≥ 60% (weight percentage), Noble = Au + Pd + Pt ≥ 25% (weight percentage).

culture but without any alloy specimens and they were used as controls.

After 72 h, cells adherent to coverslips were incubated with 10^{-6} M 5-bromodeoxyuridine (BrdU, Sigma, St. Louis, Mo, U.S.A.) for 20 min at 37°C diluted in the same culture medium. Cells were washed in phosphate buffered saline (PBS, pH 7.3), fixed in 4% paraformaldehyde in PBS (pH 7.3) for 20 min at room temperature, rinsed in PBS, denatured in 4N HCl for 30 min, dehydrated in graded ethanol (70, 90 and 100%) and air dried. Subsequently, specimens were processed for BrdU immunocytochemistry.

2.3. Determination of metal cation concentration within cell culture media

Cell culture media obtained from each culture were collected after 72 h and transferred into sterile centrifuge tubes. Concentrations (expressed in mg/mL) of Ag^{+} and Cu^{++} within these culture media were measured using a flame atomic absorption spectrophotometer (Perkin-Elmer, Wellesley, MA, USA).

2.4. BrdU immunocytochemistry

After being incubated with BrdU, slides were treated with a mouse monoclonal anti-BrdU antibody (Sigma, St. Louis, Mo, USA) diluted 1:20 in 2% bovine serum albumin (BSA) - 3% normal goat serum (NGS) in PBS, for 3 h at 37°C. Antibody binding was detected by incubating slides for 30 min at 37°C with a fluorescein isothiocyanate-conjugated (FITC) anti mouse IgG (Sigma, St. Louis, Mo, U.S.A.) diluted 1:50 in 2% BSA - 3% NGS in PBS. Slides were washed in PBS, mounted with glycerol containing 2.3% 1,4-diazobicyclo (2.2.2.) octane. For each culture sample, 10^3 cells choosen from randomly selected fields were counted always by the same operator using a Zeiss Axiophot microscope (Carl Zeiss GmbH, Jena, Germany) under both epifluorescence (wave length 490 nm) and phase contrast conditions.

The percentages of BrdU-positive cell nuclei in relationship to total nuclei number were statistically evaluated ($n = 5$, mean values and standard deviations). Statistical evaluations were performed with two-way ANOVA and Scheffe post-hoc test (statistical significance $p \leq 0.05$).

2.5. Immunocytochemistry of extracellular matrix (ECM) antigens

Fibroblast cultures that did not undergo BrdU incorporation were fixed in 4% paraformaldehyde in PBS (pH 7.3), washed in PBS and then underwent immunocytochemical reactions with antibodies recognizing the ECM consituting molecules FN and CS.

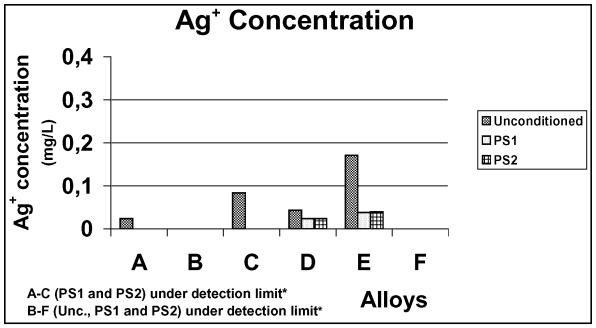
– FN-immunocytochemistry. Fibroblasts were treated with a rabbit anti-human FN antibody (Sigma, St. Louis, Mo, USA) diluted 1:100 in 4% BSA - 5% NGS in PBS and subsequently by an anti-rabbit IgG FITC-conjugated antibody (Sigma, St. Louis, Mo, USA) diluted 1:50 in 4% BSA - 5% NGS in PBS;

– CS-immunocytochemistry. Cells were incubated with a mouse monoclonal antibody (IgM) anti chondroitin-4-sulfate and chondroitin-6-sulfate (anti-CS, Sigma, St. Louis, Mo, USA) diluted 1:100 in 4% BSA - 5% NGS in PBS and subsequently with a FITC-conjugated anti-mouse IgM antibody (Sigma, St. Louis, Mo, USA) diluted 1:50 in 4% BSA - 5% NGS in PBS.

Both reactions with primary antibodies were performed after a preincubation with 4% BSA - 5% NGS in PBS to block aspecific binding sites.

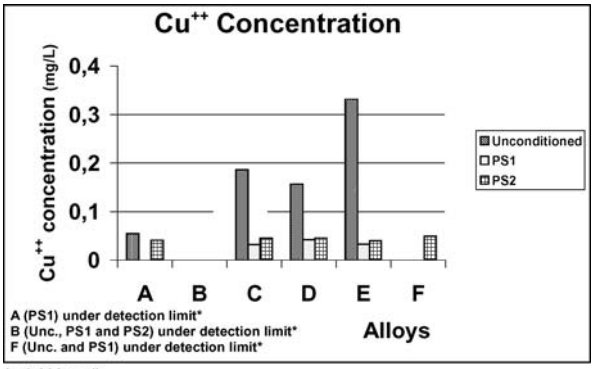
After incubation with the secondary antibodies, slides were washed in PBS and finally mounted using glycerol containing 2.3% 1,4-diazobicyclo (2.2.2.) octane. Samples were observed under epifluorescence conditions (wave length 490 nm) using a Zeiss Axiophot microscope (Carl Zeiss GmbH, Jena, Germany). Photographs were obtained on Agfachrome 100 films at the same exposure times for all specimens regarding the expression of each considered ECM antigen.

Immunofluorescence intensity was quantified as mean gray values expressed in an intensity scale ranging between 0 (black, minimum of fluorescence) and 255 (white, maximum of fluorescence) of pixels over a defined area in photographs previously converted from RBG to 8 bit gray (five areas for each sample) by using the image analysis program Optimas 6 (Optimas Corporation, Bothell, WA, USA).



* <0.024 mg/L

Figure 1 Graphic presentation of Ag^+ concentrations.



* <0,032 mg/L

Figure 2 Graphic presentation of Cu^{++} concentrations.

3. Results

3.1. Metal cations concentration within cell culture media

Cell culture media were analyzed after 72 h to determine the concentrations of metal cations Ag^+ (Fig. 1) and Cu^{++} (Fig. 2). The highest values of Ag^+ and Cu^{++} concentration were detected within cell culture media in the presence of the unconditioned alloys C, D and E. The lowest amounts of Ag^+ (i.e. below the detection limit of 0.024 mg/L) were found within cell culture media in the presence of the unconditioned alloys A, B and F, whereas the lowest Cu^{++} concentration (i.e., below the detection limit of 0.032 mg/L)were detected in the presence of the unconditioned alloys B and F.

After both preconditioning treatments of metal alloys, cell culture media in the presence of alloys C, D, E contained a decreased amount of Ag^+ and Cu^{++}, in particular in the presence of the alloy E.

After alloy preconditioning with both PS1 and PS2, cell culture media in the presence of the alloy A revealed Ag^+ concentration below its detection limit, butv also Cu^{++} concentration significantly decreased. Cell culture media in the presence of the alloy B exhibited the concentrations of both Ag^+ and Cu^{++} below their respective detection limits. Similar data (i.e. the amounts of both Ag^+ and Cu^{++} below their respective detection limit) were found within the media in the cell cultures the presence of PS1-preconditioned alloy F, whereas after the preconditioning treatment with PS2 the release of Cu^{++} from the alloy F into culture media resulted higher in comparison to both the same unconditioned metal block and after its PS1-preconditioning.

TABLE II Percentage of BrdU-positive cells after the different preconditioning treatments of metal alloys

Alloy	Without preconditioning	PS1 0.8% NaCl + 3% BSA	PS2 complete cell culture medium
A	20.17 ± 3.34^b	28.69 ± 3.22^a	27.25 ± 4.29^a
B	26.01 ± 5.44^a	28.98 ± 3.53^a	26.80 ± 3.85^a
C	20.47 ± 2.05^b	29.59 ± 3.68^a	28.65 ± 5.06^a
D	25.28 ± 4.30^a	29.05 ± 2.64^a	26.64 ± 5.81^a
E	18.37 ± 2.18^b	26.22 ± 2.72^a	28.84 ± 2.94^a
F	$26.52 \pm 3,77^a$	24.93 ± 2.18^a	26.36 ± 5.82^a
Control	27.64 ± 4.45	27.64 ± 4.45	27.64 ± 4.45

[a]Not significant.
[b]Significant ($p \leq 0.05$).

3.2. BrdU immunocytochemistry

Data concerning the immunocytochemical detection of BrdU incorporation into fibroblast nuclei are summarized in Table II. Short-term experiments of *in vitro* cytotoxicity (72 h) evidenced that the percentages of BrdU positive nuclei of fibroblasts cultured in the presence of each alloy sample pretreated with either PS1 or PS2 were similar to those of the control cultures (27.64 $\pm$ 4.45). BrdU incorporation into nuclei of fibroblasts cultured in the presence of alloys B, D and F was not statistically different in comparison to that into cells cultured in the presence of alloys pretreated with any of the preconditioning solutions, whereas when detected in cultures in the presence of both unconditioned and preconditioned alloys exhibited similar statistical data. On the contrary, fibroblasts cultured with the unconditioned alloys A (20.17 $\pm$ 3.34), C (20.47 $\pm$ 2.05) and E (18.37 $\pm$ 2.18) showed significant decreases of the proliferating cell percentage as compared both to cultures in the presence of the same alloys pretreated with PS2 and to controls ($p < 0.05$). As far as the employ of the PS1 pretreatment of the same three alloys was concerned, it was able to increase the number of BrdU positive cells only in the cultures in the presence of the alloy A ($p < 0.05$).

3.3. Immunocytochemistry of extracellular matrix (ECM) antigens

FN was less expressed in all fibroblast cultures in the presence of the six unconditioned dental metal alloys as compared to controls (Fig. 3). Only fibroblast cultures

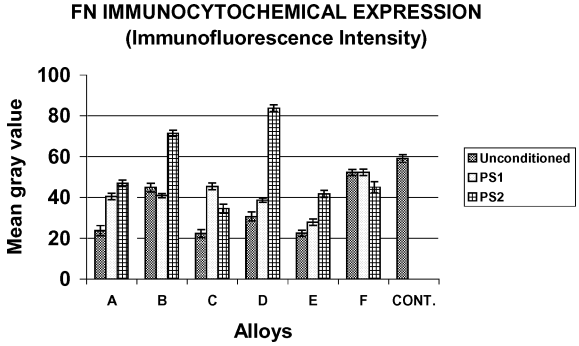


Figure 3 Graphic presentation of the quantitative evaluation of FN immunofluorescence intensity in fibroblast cultures in the presence of six single-phase dental metal alloys (A, B, C, D, E, F) both unconditioned and preconditioned with biological solutions.

in the presence of the unconditioned alloy F exhibited FN positivity similar to that expressed by control cultures, that revealed FN both within the cytoplasm and in the ECM, in which it appeared organized in fibrils.

Fibroblasts cultured in the presence of the alloys preconditioned with PS1 revealed a higher FN immunocytochemical expression in comparison to cultures in the presence of the same unconditioned alloys, even if FN positivity resulted always lower than in controls (Fig. 3). PS2-preconditioning treatment provoked a higher FN immunofluorescence in cultures in the presence of the alloys A (not statistically significant), B ($p < 0.05$), D ($p < 0.05$) and E (not statistically significant) than in cultures both in the presence of the same unconditioned alloys and after their preconditioning with PS1 (Fig. 3). FN appeared organized in fibrils and localized both within the cytoplasm and in the ECM (Fig. 4). On the contrary, fibroblasts cultured in the presence of the alloy C revealed a higher FN immunofluorescence after alloy preconditioning with PS1 in comparison to the PS2-pretreatreatment, as well as to the unconditioned biomaterial. Fibroblasts cultured in the presence of the alloy F preconditioned with PS2 exhibited a lower FN immunocytochemical expression than cultures in the presence of the unconditioned or PS1-pretreated alloy F, as well as in comparison to control cultures ($p < 0.05$).

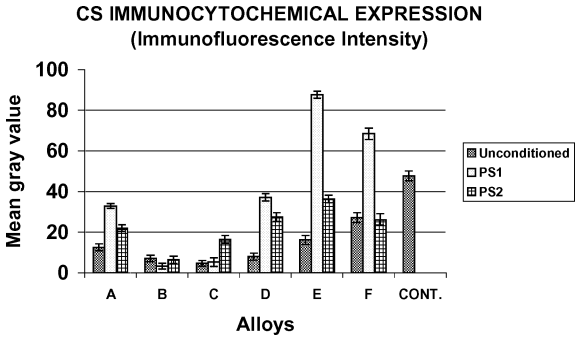


Figure 5 Graphic presentation of the quantitative evaluation of CS immunofluorescence intensity in fibroblast cultures in the presence of six single-phase dental metal alloys (A, B, C, D, E, F) both unconditioned and preconditioned with biological solutions.

CS immunocytochemical expression in fibroblast cultures in the presence of both unconditioned and PS2-preconditioned alloys appeared lower than in controls ($p < 0.05$, Fig. 5). After PS1-preconditioning treatment, cell cultures in the presence of the alloys A, D, E and F showed an increased CS immunocytochemical expression in comparison to fibroblasts

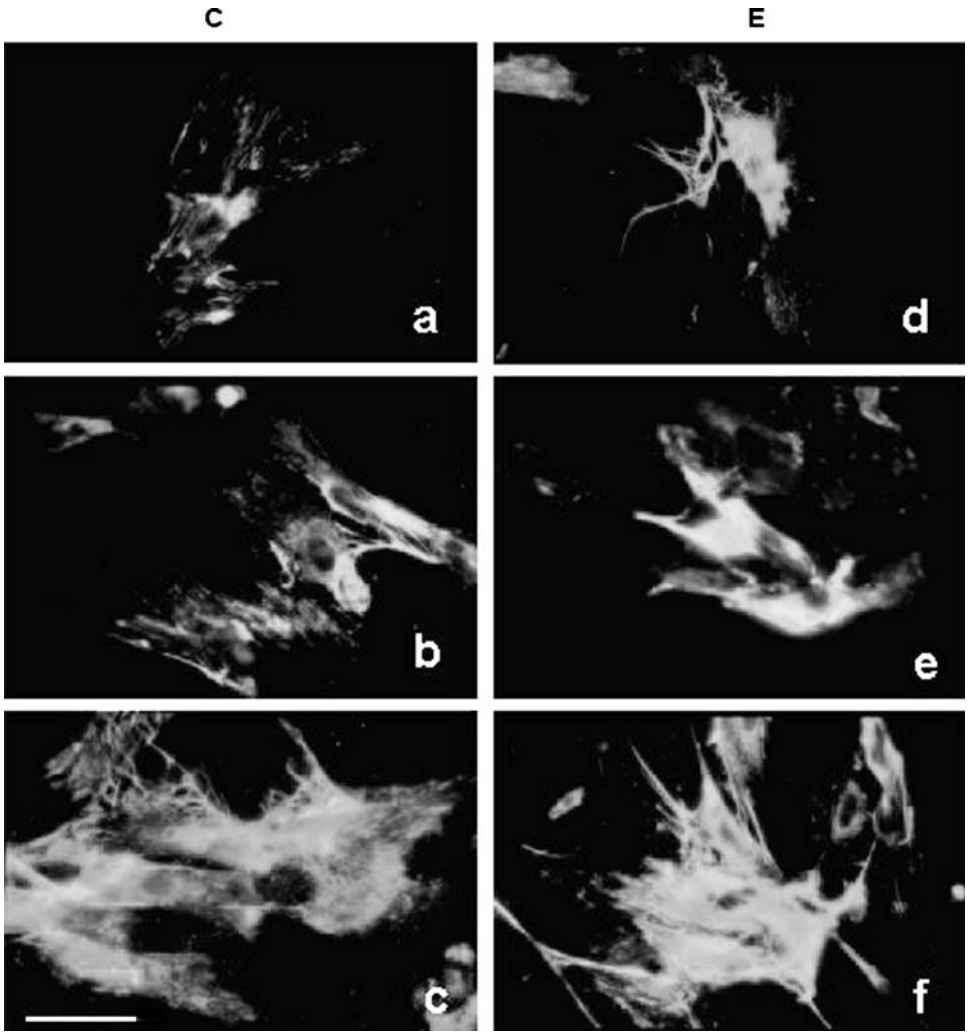


Figure 4 FN immunocytochemical expression in fibroblast cultures in the presence of two single-phase dental metal alloys (alloys C and E choosen as relevant examples): (a, d) culture in the presence of unconditioned alloys, (b, e) culture in the presence of alloys after preconditioning treatment with PS1, (c, f) culture in the presence of alloys after preconditioning treatment with PS2. Magnification bar = 40 μm.

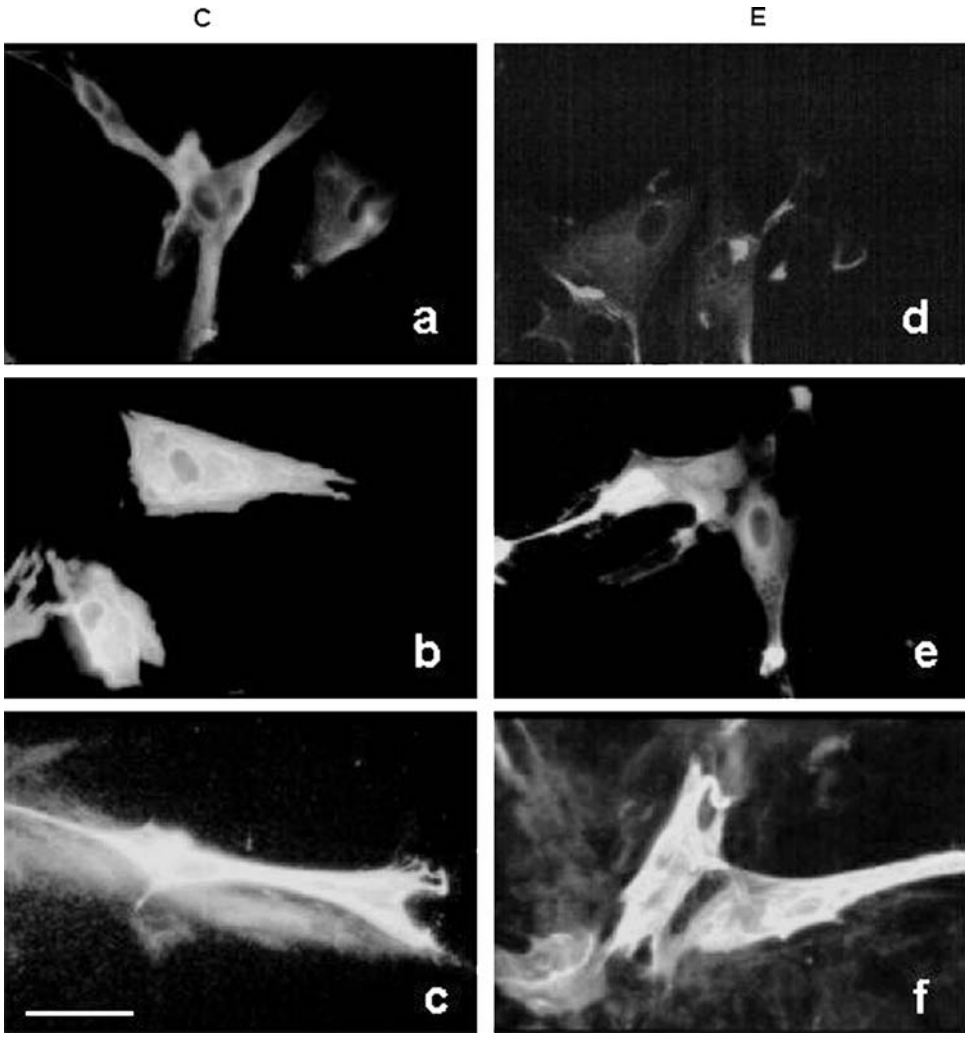


Figure 6 CS immunocytochemical expression in fibroblast cultures in the presence of two single-phase dental metal alloys (C and E choosen as relevant examples): (a, d) culture in the presence of unconditioned alloys, (b, e) culture in the presence of alloys after preconditioning treatment with PS1, (c, f) culture in the presence of alloys after preconditioning treatment with PS2. Magnification bar = 40 μm.

cultured with PS2-preconditioned alloys. Moreover, in the cultures in the presence of the alloys E and F CS-immunocytochemical positivity resulted higher also in comparison to controls, as well as CS immunocytochemical positivity was also detected within the ECM (Fig. 6). Fibroblast cultures with PS2-preconditioned alloys always revealed CS immunopositivity lower than in controls, but higher than that exhibited by fibroblasts grown in the presence of unconditioned alloys (Figs 5 and 6).

4. Discussion

Various biological parameters have been investigated over the years to evaluate the cytotoxicity biocompatibility and the biocompatibility of biomaterials, among which the evaluation of cell proliferation rates could be considered one of the most useful tools in the assessment of the biocompatibility of dental implants [21, 22, 25, 31] and dental alloys [5, 15, 24, 28–30] with a close relationship to cell adhesion capability to substrata. Moreover, previous studies *in vitro* have correlated cell proliferation rate to the expression of various ECM molecules, i.e. FN and its cellular receptor represented by the $\alpha_5\beta_1$ integrin, type I collagen and CS [21, 23, 28–31]. It has been also hypothesized that the analysis of FN organization within the ECM may represent an relevant morphological parameter to determine the biocompatibility *in vitro* of biomaterials [21, 23, 27, 28]. Moreover, the biological responses *in vitro* to the exposure to dental casting alloys has been shown to be correlated to the release of metal cations into cell culture media [20, 32] that due to the corrosion processes of these metal biomaterials: in particular, the concentrations of the most labile and, therefore, potentially cytotoxic cations, i.e., Ag^{+} and Cu^{++} [20], are strictly related to alloy biocompatibility. For this aim, the evaluation of cell proliferation rates, the organization of the ECM molecules and the release of cations into cell culture media were investigated and correlated in the present study.

Previous studies have reported that cell culture media (in particular media containing proteins) could significantly affect the corrosion properties of dental casting alloys by inducing a relevant release of ionized metal elements [32]. Nelson *et al*. have reported that preconditioning treatments of dental casting alloys with so called biological solutions could reduce alloy cytotoxicity, since such preconditioning solutions could induce different effects on the corrosion properties of dental metal alloys [20].

In the present investigation, we showed that Cu^{++} concentration was always lower in cell culture media in which fibroblasts were cultured in presence of PS1-preconditioned metal alloys in comparison to PS2-preconditioned metal biomaterials: these findings are in accordance with Nelson *et al*. [19] who found higher Cu^{++} concentration by performing the elemental analysis of a medium employed in alloy preconditioning treatment and containing NaCl and BSA, whose composition was very similar to the PS1 proposed by us.

We also observed that the alloy F released a higher amount of Cu^{++} into cell culture media when preconditioned with PS2 as compared to the same unconditioned biomaterial and to the preconditioned one with PS1. This fact would seem contradictory, but it should be related to the specific alloy F composition that could delay its corrosion process after the test period of 72 whenever it underwent PS2-preconditioning treatment.

As far as the release of Ag^{+} into cell culture media was concerned, it was similarly influenced by alloy preconditioning treatment with either PS1 or PS2. Indeed, both these biological solutions provoked the decrease of Ag^{+} concentration within cell culture media, therefore it is reasonable to assume that both PS1 and PS2 could be considered useful tools to remove Ag^{+} from metal alloys.

We could observe that both alloy preconditioning treatments influenced the biological behavior of fibroblast cultures. In particular, cell proliferation rates increased as revealed by higher percentages of BrdU-positive within cultures in the presence of the preconditioned dental alloys in comparison to those with the unconditioned biomaterials. This evidence, together with the decreased metal cation concentrations within culture media, could confirm the utility of alloy preconditioning treatments, in accordance to Nelson *et al*. [20], who also confirmed a higher cell viability in cell cultures in the presence of preconditioned alloys.

Cell proliferation rates in the presence of the unconditioned alloys A, C and E were significantly different as compared to control cultures; on the contrary, no significant differences of cell proliferation rate were detected in cultures with the unconditioned alloys B, D and F in comparison to control. These evidences should lead to the conclusion that cell proliferation rates of fibroblast cultures with unconditioned high noble alloys (A, C, E) were significantly different to controls (i.e., cell cultures without any alloy), whereas preconditioning treatments with either PS1 and PS2 induced a biological behavior similar to control.

In accordance to previous reports [21, 23, 28–30], cell proliferation rates were correlated to the immunocytochemical expression of some ECM antigens, i.e. FN and CS. Data obtained in the present investigation confirmed that higher FN immunofluorescence corresponded to higher percentages of BrdU-positive cells. In particular, a relevant FN immunofluorescence was observed in cultures with alloys A, B, C, D, E that were previously pretreated with PS1, whereas only fibroblast cultures in the presence of PS1-preconditioned alloy F revealed a weaker FN immunofluorescence intensity. The preconditioning treatment with PS2 induced an increasing expression of FN in comparison to cells cultured together PS1-preconditioned metal blocks. These data are in accordance with those regarding cell proliferation rates and metal cation concentrations within cell culture media, therefore, we could assume and confirm that evaluation of FN expression could be considered a useful tool to assess the biocompatibility *in vitro* of dental metal alloys.

As far as the immunocytochemical expression of CS was concerned, it was more influenced by alloy pretreatment with PS1 than with PS2. This finding was particularly evident in the presence of the alloys E and F preconditioned with PS1 in comparison to the same metal specimens pretreated with PS2.

5. Conclusions

On the bases of results obtained by performing the present investigation, we may assume that the biocompatibility of dental metal alloys could be usefully evaluated *in vitro* by using models of human cell cultures and by correlating metal cation release into cell culture media both to cell proliferation rates and to the immunocytochemical expression of ECM molecules, i.e., FN and CS. We could also affirm that the employ of preconditioning treatments of dental metal alloys with biological solutions could enhance their biocompatibility, since more labile metal elements could be removed from dental metal alloys in form of cations, thus avoiding or limiting their successive release either into cell culture media during evaluations *in vitro*, or into biological fluids, i.e., saliva, during evaluations *in vivo* in laboratory animals, as well as in clinical applications. Therefore, the use of preconditioning treatments should be recommended not only before tests of biocompatibility *in vitro*, but it could be suggested also before any clinical employ of dental metal alloys.

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References

1. S. SYRJÄNEN, A. HENSTEN-PETTERSEN, K. KANGASNIEMI and A. YLI-URPO, *Biomaterials* **6** (1985) 169.
2. D. BRUNE, *ibid.* **7** (1986) 163.
3. J. C. WATAHA, *J. Prosthet. Dent.* **83** (2000) 223.
4. R. G. CRAIG and C. T. HANKS, *J. Dent. Res.* **69** (1990) 1539.
5. J. C. WATAHA, R. G. CRAIG and C. T. HANKS, *ibid.* **70** (1991) 1014.
6. J. C. WATAHA, C. T. HANKS and R. G. CRAIG, *J. Biomed. Mater. Res.* **26** (1992) 1297.
7. J. D. BUMGARDNER, L. C. LUCAS, M. W. JR. ALVERSON and A. B. TILDEN, *Dental Mater.* **9** (1993) 85.
8. J. D. BUMGARDNER, J. DOELLER and L. C. LUCAS, *J. Biomed. Mater. Res.* **29** (1995) 611.
9. J. D. BUMGARDNER and L. C. LUCAS, *J. Dent. Res.* **74** (1995) 1521.
10. J. C. WATAHA, C. T. MALCOLM and C. T. HANKS, *Int. J. Prosthodont.* **8** (1995) 9.
11. J. C. WATAHA, P. E. LOCKWOOD and A. SCHEDLE, *J. Biomed. Mater. Res.* **52** (2000) 360.

12. A. WENNEBERG, I. A. MJÖR and A. HENSTEN-PETTERSEN, *ibid.* **17** (1983) 23.
13. L. NIEMI and A. HENSTEN-PETTERSEN, *ibid.* **19** (1985) 549.
14. R. G. CRAIG and C. T. HANKS, *J. Oral. Pathol.* **17** (1988) 341.
15. J. C. WATAHA and P. E. LOCKWOOD, *Dental Mater.* **14** (1998) 158.
16. J. C. WATAHA, P. E. LOCKWOOD and S. K. NELSON, *J. Oral. Rehabil.* **26** (1999) 798.
17. J. C. WATAHA, S. K. NELSON and S. BOUILLAGUET, *Int. J. Prosthodont.* **12** (1999) 242.
18. J. C. WATAHA, P. E. LOCKWOOD, S. K. NELSON and D. RAKICH, *J. Oral Rehabil.* **26** (1999) 379.
19. S. K. NELSON, J. C. WATAHA, A. M. L. NEME, R. M. CIBIRKA and P. E. LOCKWOOD, *J. Prosthet. Dent.* **81** (1999) 591.
20. S. K. NELSON, J. C. WATAHA and P. E. LOCKWOOD, *ibid.* **81** (1999) 715.
21. V. GRILL, M. A. SANDRUCCI, M. BASA, R. DI LENARDA, E. DORIGO, A. M. MARTELLI, R. BAREGGI and P. NARDUCCI, *J. Biol. Res.* **72** (1996) 87.
22. M. KÖNÖNEN, J. HORMIA, J. KIVILAHTI, J. HAUTANIEMI and I. THESLEFF, *J. Biomed. Mater. Res.* **26** (1992) 1325.
23. V. GRILL, M. A. SANDRUCCI, M. BASA, R. DI LENARDA, E. DORIGO, A. M. MARTELLI, R. BAREGGI and P. NARDUCCI, *Archs. Oral Biol.* **42** (1997) 641.
24. M. AHMAD, M. B. MCCARTHY and G. GRONOWICZ, *Biomaterials* **20** (1999) 211.
25. J. H. VAN DE DIERENDOCK, R. KEYZER, C. J. H. VAN DE VELDE and C. J. CORNELISSE, *Citometry* **10** (1989) 143.
26. L. M. NERI, G. MAZZOTTI, S. CAPITANI, N. M. MARALDI, C. CINTI, N. BALDINI, R. RANA and A. M. MARTELLI, *Histochemistry* **98** (1992) 19.
27. V. GRILL, M. A. SANDRUCCI, R. DI LENARDA, M. BASA, P. NARDUCCI, A. M. MARTELLI and R. BAREGGI, *Quintessence Int.* **31** (2000) 741.
28. V. GRILL, M. A. SANDRUCCI, R. DI LENARDA, M. CADENARO, P. NARDUCCI, R. BAREGGI and A. M. MARTELLI, *J. Biomed. Mater. Res.* **52** (2000) 479.
29. V. GRILL, M. A. SANDRUCCI, R. DI LENARDA, E. DORIGO, P. NARDUCCI, A. M. MARTELLI and R. BAREGGI, *J. Oral Sci.* **42** (2000) 1.
30. J. C. WATAHA, S. K. NELSON and P. E. LOCKWOOD, *Dental Mater.* **17** (2001) 409.

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