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Comparison of in vitro cytotoxicity and genotoxicity of MMA-based polymeric materials and various metallic materials

Sultan GÜLÇE İZ[1], Saime İsmet DELİLOĞLU GÜRHAN[1], Bilge Hakan ŞEN[2], Tuğba ENDOĞAN[3], Nesrin HASIRCI[3,4]

Aim: To determine the in vitro cytotoxicity and genotoxicity of some polymeric and metallic implant materials used as base materials in dentistry, based on ISO (International Organization for Standardization) and OECD (Organization for Economic Co-Operation and Development) test protocols.

Materials and methods: Three different acrylate-based polymeric materials were tested for their in vitro cytotoxicity and genotoxicity (polymethylmethacrylate microspheres [PMMA], a solid cement prepared by mixing PMMA with its monomer methylmethacrylate [PMMA+MMA], a solid cement prepared by mixing PMMA, MMA, and hydroxyapatite [PMMA+MMA+HA], as wells as 4 different metallic materials (titanium [Ti grade 4], nickel alloy 625 [Ni-625], stainless steel alloy 304L [SS-304L], and stainless steel alloy 321 [SS-321]).

Cytotoxic effects of the materials were determined using L929 mouse fibroblasts by MTT assay. Cell attachment properties related to the biocompatibility of the materials were analyzed using a scanning electron microscope (SEM). Genotoxicity of the materials was determined with human peripheral lymphocytes via micronucleus assay.

Results: The highest compatibility was exhibited by Ti grade 4, followed by Ni-625, SS-304L and, SS-321. Among the polymeric materials, PMMA+MMA+HA had the highest biocompatibility, followed by PMMA+MMA and PMMA.

Conclusion: The biocompatibility of the metallic materials was higher than that of the polymeric materials. Ti, the most inert metal, exhibited the highest biocompatibility. The addition of HA reduced the cytotoxic and mutagenic effects of MMA monomer and leachable ingredients.

Key words: Biocompatibility, in vitro cytotoxicity, in vitro genotoxicity

MMA tabanlı polimerik materyallerin in vitro sitotoksisite ve genotoksisitesinin çeşitli metalik materyallerle karşılaştırılması

Amaç: Bu çalışmanın amacı, özellikle diş hekimliğinde kullanılan polimerik ve metalik malzeme tabanlı implant materyallerinin in vitro sitotoksisite ve genotoksisitesinin ISO (Uluslararası Standardizasyon Organizasyonu) ve OECD (Ekonomik İşbirliği ve Gelişim Organizasyonu) protokollerine göre belirlenmesidir.

Yöntem ve gereç: Bu çalışmada, üç farklı akrilik tabanlı polimerik materyal; polimetilmetakrilat mikroküreleri (PMMA); PMMA ve monomeri metilmetakrilat (MMA) ile karışımından oluşan katı çimento, PMMA, MMA ve hidroksiapetit (HA) karışımından oluşan katı çimento ve dört farklı metalik materyal; titanyum (Ti grade 4), nikel alaşımı 625 (Ni-625), iki paslanmaz çelik alaşımı, alaşım 304L (SS-304L) ve alaşım 321 (SS-321) in vitro sitotoksisite ve genotoksisite özellikleri bakımından test edilmiştir. Materyallerin sitotoksik etkileri L929 fare fibroblast hücreleri kullanılarak MTT testi ile belirlenmiştir. Biyouyumlulukla birebir ilişkili olan materyal yüzeyine hücre tutunma özellikleri ise taramalı elektron mikroskobi (SEM) ile analiz edilmiştir. Materyallerin genotoksisiteleri ise insan periferal lenfositleri kullanılarak yapılan mikronükleus testi ile belirlenmiştir.

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[1] Department of Bioengineering, Faculty of Engineering, Ege University, İzmir - TURKEY,
[2] Department of Endodonty, Faculty of Dentistry, Ege University, İzmir - TURKEY,
[3] Department of Polymer Science and Technology, Middle East Technical University, Ankara - TURKEY
[4] Department of Chemistry, Middle East Technical University, Ankara - TURKEY

Correspondence: *S. İsmet DELiLOĞLU GÜRHAN, Ege University, Faculty of Engineering, Department of Bioengineering, İzmir - TURKEY*
E-mail: s.ismet.gurhan@ege.edu.tr

Bulgular: En yüksek biyouyumluluk Ti-grade4'de gözlenmiş Ni-625, SS-304L ve SS-321 sırasıyla bu materyali izlediği gösterilmiştir. Polimerik materyaller arasında ise PMMA+MMA+HA en yüksek biyouyumluluğu göstermekte iken sırasıyla PMMA+MMA ve PMMA onu izlemektedir.

Sonuç: Metalik materyallerin biyouyumlulukları polimerik olanlardan daha yüksek bulunmuştur. Ti'un inert bir materyal oluşu biyouyumluluk özelliğini desteklemektedir. Bileşiğin içerisine HA ilavesi MMA monomerinin sitotoksik ve mutajenik etkilerini ve bileşikten salınması muhtemel monomer oranını azaltmıştır.

Anahtar sözcükler: Biyouyumluluk, in vitro sitotoksisite, in vitro genotoksisite

Introduction

Metals and polymers are commonly used in dental and orthopedic applications to restore damaged hard tissue. As the biocompatibility of any material used in the human body is a crucial property, tests to ensure the biological compatibility of materials have been specified by regulatory organizations (1).

Metals and alloys are commonly used dental materials because of their high strength and other desirable properties. The base metal alloy systems commonly used in dentistry are stainless steel, nickel-chromium, cobalt-chromium, titanium, and nickel-titanium alloys. Certain base metal alloy systems are preferred because of their superior mechanical properties, low density, and, in some cases, their ability to osseointegrate (2).

In addition, a variety of different polymeric materials and composites are used in dentistry. Polymeric dental materials are based on methacrylate, its polymer, and polyelectrolytes. Composites used for filling teeth are generally made of silica or glass particles bound with a polymer resin. The polymers that are used as the resin in composites for fillings are primarily based on acrylate monomers. Both metallic alloys and polymers have several disadvantages, such as cytotoxicity. In general, cytotoxicity due to metallic materials is the result of corrosion and the release of metal ions. Residual monomers or oligomers formed by the degradation processes that are released or are present in the structure because of incomplete polymerization are considered the cause of polymeric cytotoxicity (2,3).

Biocompatibility of materials is increasingly evaluated using cultured cells because of their lower cost, shorter test period, and higher reproducibility and reliability, as compared to in vivo evaluation, in addition to ethical considerations (4). Pure metals and alloys, metal salts, particulate metals and metallic debris, dental composite materials, root canal sealers, dental resinous cements, dental adhesives, glass ionomer cements, etc. have all been tested for their cytotoxicity (1,4-8). The present study aimed to test several metallic and polymeric materials used in dental applications as base materials, based on ISO and OECD test protocols (9-12).

Materials and methods

Test materials, chemicals, and reagents

Metallic samples were obtained from ZAPP (USA) as metal plates; the chemical composition of each metallic material is given in Table 1.

For the preparation of polymeric samples the following materials were used: methyl methacrylate monomer (MMA), polyvinyl alcohol (PVA) (both obtained from Acros Organics, USA), N,N-dimethyl-p-toluidine (DMPT), benzoyl peroxide (BPO) (both obtained from Sigma-Aldrich Chemie, Germany), hydroxyapatite (HA) (obtained from Riedel-de Haën A.G., Germany), sodium hydroxide (NaOH) (a product of J. T. Baker, Holland), and barium sulphate ($BaSO_4$) (obtained from Merck, Germany). All chemicals, except MMA, were used as obtained without further purification. MMA contains hydroquinone as an inhibitor to prevent premature polymerization; therefore, prior to the polymerization reaction it was washed with 10% wt aqueous sodium hydroxide solution to remove the inhibitor. The composition of each polymeric material used in this study is listed in Table 2.

L929 mouse connective tissue fibroblasts obtained from HUKUK (Foot and Mouth Disease Institute, Animal Cell Culture Collection, Ankara, Turkey) were used for cell culture tests. Cell culture medium (RPMI 1640), phytohemagglutinin (PHA), L-

Table 1. Chemical composition of metallic materials.

Metal Type/Composition	Ni-625	SS-321	SS-304L	Ti Grade 4
C%	0.05	0.08	0.08	0.08
Mn%	0.030	2.00	2.00	–
P %	0.010	0.045	0.045	–
S %	0.003	0.030	0.030	–
Si%	0.25	0.75	0.75	–
Cr%	22.0	17.0/19.0	18.0/20.0	–
O%	–	–	–	0.4
N%	–	0.10	0.10	0.10
Ni %	Equilibrium	9.0/12.0	8.0/10.5	–
Mo%	9.0	–	–	–
Ta%	3.5	–	–	–
Ti%	0.3	5 × (C+N)	–	Equilib-
rium				
Al%	0.3	–	–	–
Fe%	4.0	Equilibrium	Equilibrium	0.5
H%	–	–	–	0.01
Co%	–	–	–	–

Table 2. Chemical composition of polymeric materials.

Polymer Type/Composition	PMMA	PMMA+MMA	PMMA+MMA+HA
Powder Part	PMMA; 4 g	PMMA; 4 g BPO; 45 mg $BaSO_4$; 604 mg	PMMA; 4 g BPO; 45 mg $BaSO_4$; 604 mg HA; 348 mg
Liquid Part	–	MMA; 6 mL DMPT; 56 µL	MMA; 6 mL DMPT; 56 µL

glutamine, gentamycin, and fetal bovine serum (FBS) were purchased from Biochrom (Germany). Trypsin, MTT [3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide], and DAPI (4,6-diamidino-2-phenylindole) were purchased from Sigma (Germany). Trypan blue, DMSO (dimethylsulfoxide), and mitomycin C were purchased from Applichem (Germany). Cytochalasin B was purchased from MP Biomedicals Inc. (France).

Extraction conditions

Polymeric material samples were prepared in glass rings (6 mm in diameter and 1 mm high). Metallic materials were cut into squares (10-mm edge length and 0.25-mm height). Extracts of these samples were prepared following the recommendations of ISO 10993-12 at a ratio of 117.8 mm^2 of sample surface area/mL of cell culture medium at 37 °C and 5% CO_2 for a 72-h extraction period (13).

In vitro cytotoxicity test

L929 mouse connective tissue fibroblasts were routinely cultivated in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C and 5% CO_2. Into each well of a 24-well plate, 8×10^4 cells were seeded and incubated

for 24 h at 37 °C. After overnight cultivation, the culture medium was replaced with fresh medium that contained serial dilutions of the extracts of the test specimens. Non-treated cell culture and zinc extract were used as positive and negative controls, respectively. After 48 h the exposure medium was discarded. Cell viability after exposure was determined using the MTT assay, which is a colorimetric test that measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium to a purple formazan product (14). The absorbance of viable cells was immediately determined at 570 nm using a UV-visible single beam spectrophotometer (Jenway 6400, UK).

Cell morphology

SEM micrographs of the metallic and polymeric samples were taken 24 h after cell attachment in order to examine the morphology (primarily the shape and attachment properties of the L 929 cells). The samples were prepared according to the SEM protocol, coated with a thin layer of gold via ion sputtering, and examined using a Jeolism-5200 SEM (Tokyo, Japan) (15).

In vitro micronucleus assay

In vitro micronucleus assay was carried out with human peripheral blood lymphocytes. Blood from 2 healthy, young (less than 35 years of age) non-smoking donors were used, without pooling. Lymphocytes were obtained from volunteers with the approval of the Ege University Medical Faculty Research Ethics Committee. Blood cells were cultivated with 20 µg/mL of PHA-supplemented cell culture medium at 37 °C and 5% CO_2. After 24 h the cell culture medium was removed and the cells were exposed to 5 mL of serially diluted material extracts for 48 h. As a positive control, 0.10 µg/mL of mitomycin C was used. Untreated lymphocytes without PHA were used for the blank control. At the 44^{th} hour of the culture period, 6 µg/mL of cytochalasin B was added to the culture tubes to inhibit microfilament assembly and cytokinesis, and thus to prevent the separation of daughter cells after mitosis leading to binucleated cells. After 72 h the cells were fixed onto microscope slides and air dried. Staining was performed with 5% Giemsa and 1 µg/mL of DAPI (12). The number of micronuclei was analyzed microscopically in 1000 cells/slide of 3 parallel cultures (slides) per concentration in 3 independent experiments, as described in Table 4.

Statistical analysis

For in vitro cytotoxicity assay 3 replicates of each concentration were performed for each test; the tests were repeated 3 times to ensure reproducibility. The significance of differences between the groups was statistically analyzed by one-way variance analysis using Prism 5.0 (Graphpad, USA). Repeated measures of ANOVA was followed by Bonferroni's multiple comparison post-hoc test, and a P value less than 0.05* was considered statistically significant. For in vitro micronucleus assay a P value less than 0.001* was considered statistically significant.

Results

In vitro cytotoxicity tests: Cell viability

Cytotoxicity tests were carried out in triplicate using various dilutions of the extracts. The absorbance of viable cells for each dilution determined using the MTT assay is shown in Figure 1. Statistically significant differences between the cell control group and the material extracts-treated groups are shown in Table 3. The absorbance of viable cells was converted into a percentage, assuming that cell control absorbance was 100% viability. Viability (%) of the cells exposed to the metallic and polymeric material extracts is shown in Figures 2 and 3.

Zinc extract was used as a negative (-) control because of the well-known high cytotoxic effect of zinc (16,17). Extracts of zinc inhibited cell growth at dilutions of 1/1, ½, and ¼ below 50% of the cells, but at 1/8 dilution the viable cell ratio was higher than 50%, as shown in Figure 3.

No significant differences between the cytotoxicity of the metallic materials at each dilution were observed ($P > 0.05$). When evaluating means Ti grade 4 exhibited the highest cell viability (99%), followed by Ni-625 (98%), SS-304 L (97%), and SS-321 (95%), as shown in Figure 2. Evaluation of the serial material extract dilutions showed that there were no significant ($P > 0.05$) differences between the cytotoxicity of the different metallic material extracts at all dilutions for which the viability ratio was above 95% (Figures 1 and

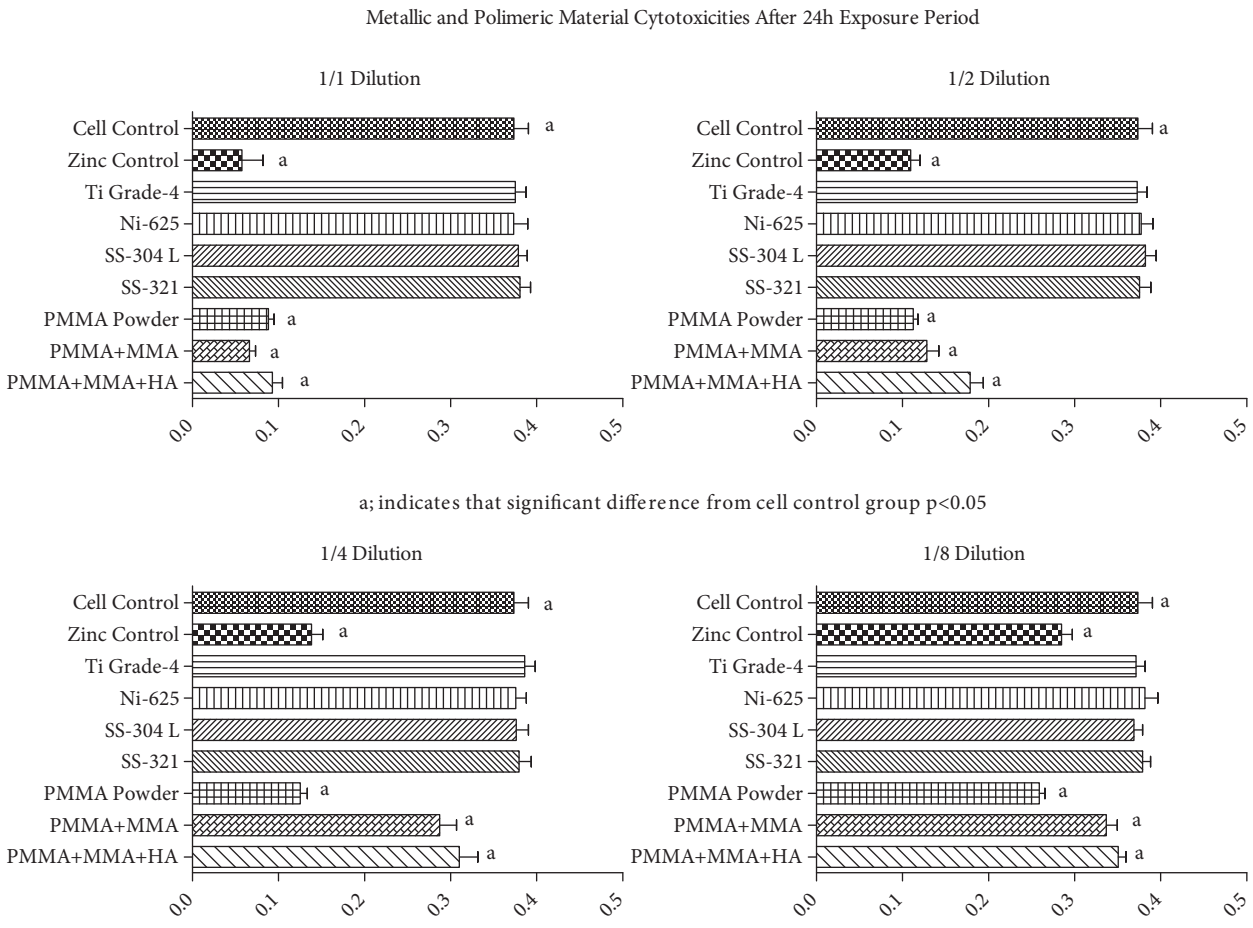

Figure 1. Metallic and polymeric material cytotoxicities after 24-h exposure.

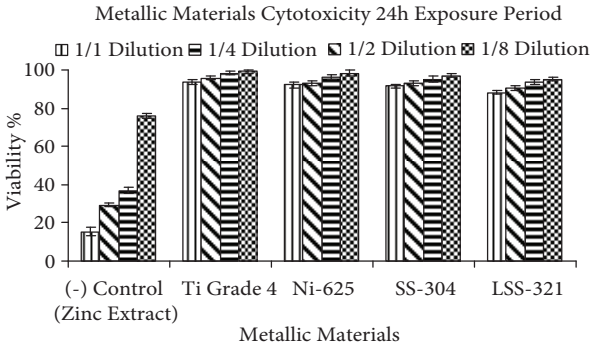

Figure 2. Percentage cell viability of the metallic materials after 24-h exposure.

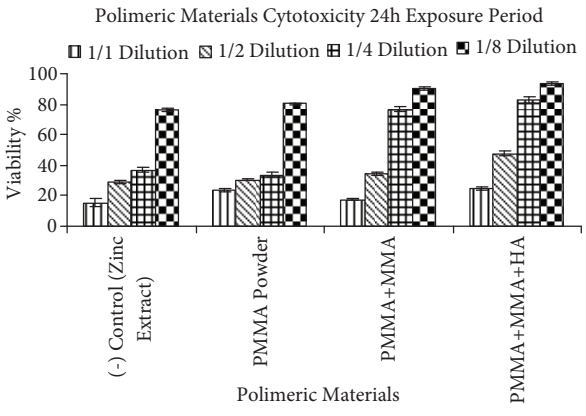

Figure 3. Percentage cell viability of the polymeric materials after 24-h exposure.

2, and Table 3), which indicates that the metallic materials are highly biocompatible.

Among the polymeric samples, significant differences were observed between sample groups and all dilutions ($P < 0.05$), as shown in Figure 1 and Table 3. PMMA powder extract exhibited a strong cytotoxic effect at dilutions of 1/1 and ½, which indicates that the PMMA+MMA+HA polymer had the least cytotoxic effect. Among the polymeric materials, PMMA+MMA+HA had the highest cell viability (93%), followed by PMMA+MMA (90%), and PMMA (80%), as shown in Figure 3. Evaluation of the serial dilutions of the material extracts showed that the maximum viability rates were observed at 1/8 dilutions of each material extract.

Table 3. The statistical difference between the groups ($P < 0.05$).

One-way ANOVA followed by Bonferroni's multiple comparison test	1/1 Dilution	1/2 Dilution	1/4 Dilution	1/8 Dilution
Cell Control vs Zinc Control	***	***	***	***
Cell Control vs Ti Grade-4	ns	ns	ns	ns
Cell Control vs Ni-625	ns	ns	ns	ns
Cell Control vs SS-304 L	ns	ns	ns	ns
Cell Control vs SS-321	ns	ns	ns	ns
Cell Control vs PMMA Powder	***	***	***	***
Cell Control vs PMMA+MMA	***	***	***	***
Cell Control vs PMMA+MMA+HA	***	***	***	***
Zinc Control vs Ti Grade-4	***	***	***	***
Zinc Control vs Ni-625	***	***	***	***
Zinc Control vs SS-304 L	***	***	***	***
Zinc Control vs SS-321	***	***	***	***
Zinc Control vs PMMA Powder	***	ns	ns	***
Zinc Control vs PMMA+MMA	ns	ns	***	***
Zinc Control vs PMMA+MMA+HA	***	***	***	***
Ti Grade-4 vs Ni-625	ns	ns	ns	ns
Ti Grade-4 vs SS-304 L	ns	ns	ns	ns
Ti Grade-4 vs SS-321	ns	ns	ns	ns
Ti Grade-4 vs PMMA Powder	***	***	***	***
Ti Grade-4 vs PMMA+MMA	***	***	***	***
Ti Grade-4 vs PMMA+MMA+HA	***	***	***	***
Ni-625 vs SS-304 L	ns	ns	ns	ns
Ni-625 vs SS-321	ns	ns	ns	ns
Ni-625 vs PMMA Powder	***	***	***	***
Ni-625 vs PMMA+MMA	***	***	***	***
Ni-625 vs PMMA+MMA+HA	***	***	***	***
SS-304 L vs SS-321	ns	ns	ns	ns
SS-304 L vs PMMA Powder	***	***	***	***
SS-304 L vs PMMA+MMA	***	***	***	***
SS-304 L vs PMMA+MMA+HA	***	***	***	ns
SS-321 vs PMMA Powder	***	***	***	***
SS-321 vs PMMA+MMA	***	***	***	***
SS-321 vs PMMA+MMA+HA	***	***	***	***
PMMA Powder vs PMMA+MMA	ns	ns	***	***
PMMA Powder vs PMMA+MMA+HA	ns	***	***	***
PMMA+MMA vs PMMA+MMA+HA	***	***	ns	ns

***; indicates significant differences between groups; $P < 0.05$. ns; non-significant.

One of the properties of surfaces that determine the suitability for cell attachment is the cytotoxicity of the surface material. SEM photographs of the cells are shown in Figures 4 and 5.

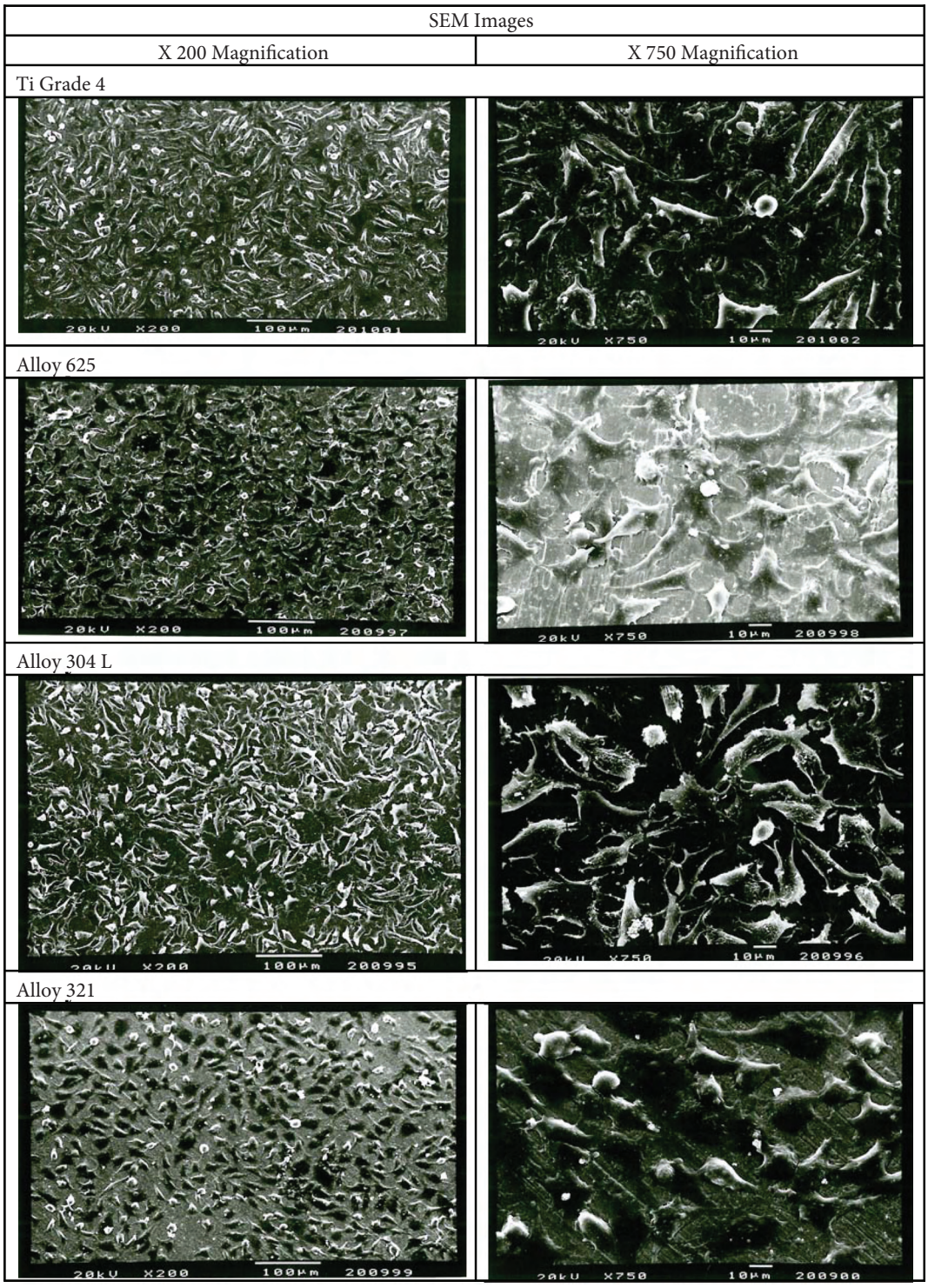

Figure 4. SEM images of the cells on metallic material surfaces.

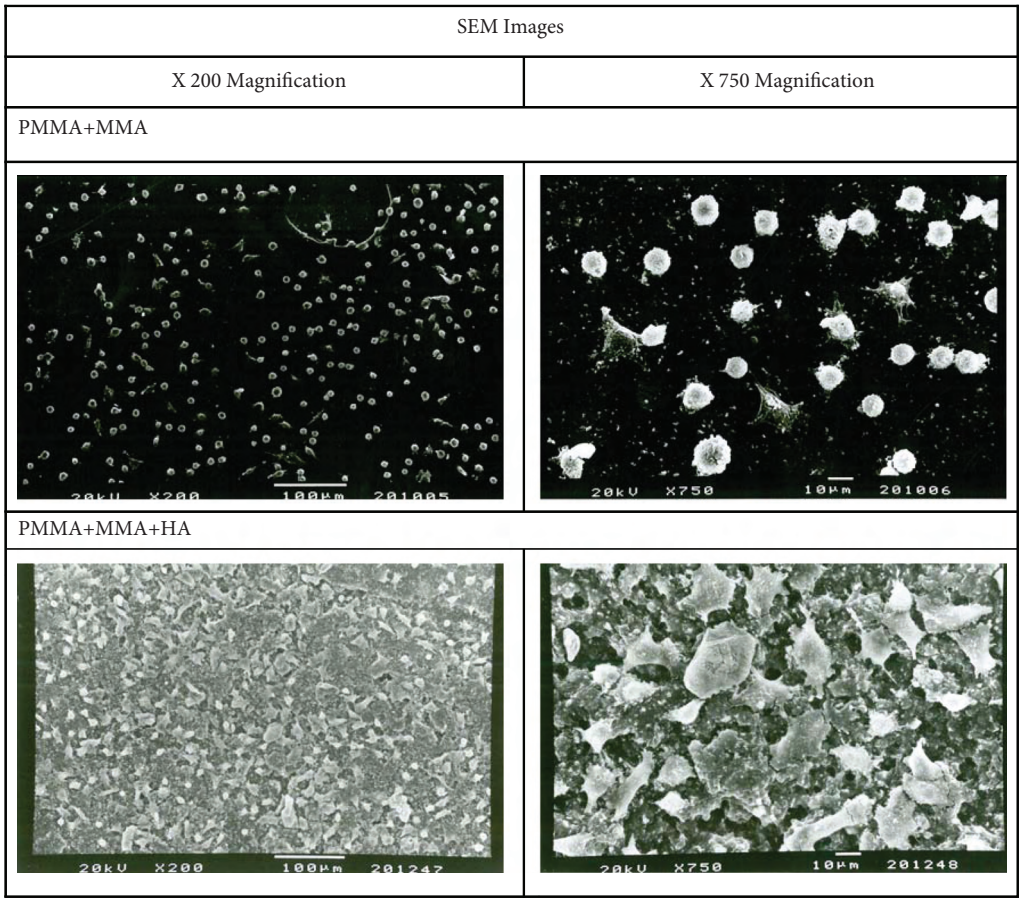

Figure 5. SEM images of the cells on polymeric material surfaces.

The cells attached to the surfaces of each metallic material properly, with fibroblastic morphologies and a spreading nature. There were no significant morphological differences between the metallic materials, as with the cell viability test results. As PMMA is a powder, it could be used for attachment tests. As seen at 200× and 750× magnifications, the PMMA+MMA+HA polymeric surface was more suitable for cell attachment than was the PMMA+MMA surface. Cells on the PMMA+MMA+HA surface were confluent and spread over the entire material surface, and were likely to exhibit typical fibroblastic features; however, cell confluence on the PMMA+MMA surface was low, with less spreading.

In vitro genotoxicity tests

Micronucleus tests were performed in triplicate; values given in Table 4 are the average of 3 experiments. The dilutions of the material extracts that caused 50% cell survival (IC_{50} values, half the maximum inhibitory concentration) were calculated. The IC_{50} value for zinc, PMMA powder, and PMMA+MMA extracts was 1/8, versus 1/4 for the PMMA+MMA+HA extract and 1/1 for the metallic material extracts. Micronucleus tests were conducted only with these dilutions. Giemsa and DAPI staining of the cells with micronuclei are shown in Figures 6 and 7.

In the present study MN evaluation was performed with binucleated cells in which cytokinesis was blocked by cytochalasin B. As the cytokinesis block technique was used, the frequency of binucleated cells with micronuclei (and with 1, 2, and more than 2 micronuclei) was determined. In the samples treated with mitomycin C and zinc extract (positive controls) 29.7% and 12.7% of cells had 1

Table 4. Percentage of micronuclei of the cells exposed to the metallic and polymeric material extracts.

Micronucleus (MN) Assay Micronucleus Count / 1000 cells	Uninuclear Cells				Binuclear Cells			
	Without MN	1 MN	2 MN	>3 MN	Without MN	1 MN	2 MN	>3 MN
Cell Culture Medium Control	504/1000 50.4%	8/1000 0.8%	- 0%	- 0%	472/1000 47..2%	16/1000 1.6%	- 0%	- 0%
MCM C (Mytomicin C) (+) Control	257/1000 25.7%	204/1000 20.4%	40/100 4%	20/1000 2%	30/1000 3%	297/1000 29.7%	146/1000 14.6%	6/1000 0.6%
Zinc (+) Control	450/1000 45%	16/1000 1.6%	- 0%	- 0%	400/1000 40%	129/1000 12.9%	5/1000 0.5%	- 0%
PMMA	470/1000 47%	20/1000 2%	- 0%	- 0%	402/1000 40.2%	101/1000 10.1%	7 0.7%	- 0%
PMMA + MMA	441/1000 44.1%	28/1000 2.8%	- 0%	- 0%	442/1000 44.2%	83/1000 8.3%	6 0.6%	- 0%
PMMA+MMA+HA	460/1000 46%	25/1000 2.5%	- 0%	- 0%	461/1000 46.1%	47/1000 4.7%	7 0.7%	- 0%
Ti Grade 4	510/1000 51%	16/1000 1.6%	- 0%	- 0%	456/1000 45.6%	18/1000 1.8%	- 0%	- 0%
Alloy 625	500/1000 50%	20/1000 2%	- 0%	- 0%	452/1000 44%	28/1000 2.8%	- 0%	- 0%
Alloy 304 L	446/1000 44.6%	19/1000 1.9%	- 0%	- 0%	500/1000 50%	35/1000 3.5%	- 0%	- 0%
Alloy 321	420/1000 42%	25/1000 2.5%	- 0%	- 0%	513/1000 51.3%	42/1000 4.2%	- 0%	- 0%

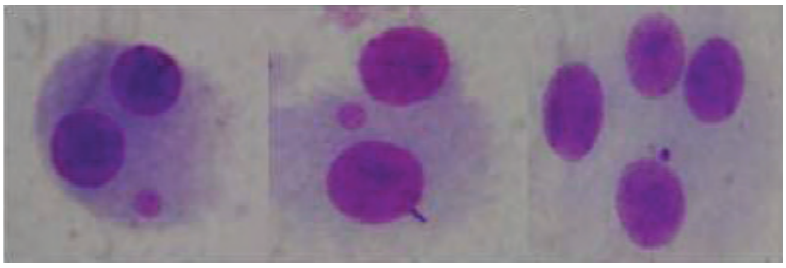

Figure 6. Giemsa staining of the cells with micronuclei.

Figure 7. DAPI staining of the cells with micronuclei.

MN, and 14.6% and 0.5% had 2 MN, respectively; 3 MN was not observed in the samples treated with zinc extract, but was observed in 0.6% of those treated with mitomycin C. In the present study the frequency of MN formation in the metallic materials Ti grade 4, alloy 625, alloy 304L, and alloy 321 was 1.8%, 2.8%, 3.5%, and 4.2%, respectively, and the differences between them were significant ($P < 0.001$). Cells with 2 micronuclei were not observed in metallic material extracts, only cells with 1 micronucleus were observed. Evaluation of the frequency of MN formation in the polymeric material extracts showed that the sum of 1 MN and 2 MN cells in PMMA, PMMA+MMA, and PMMA+MMA+HA was 10.8%, 8.9%, and 5.4%, respectively, much higher than that observed in the metallic material extracts.

Discussion

Metals, alloys, and polymers are the most commonly used dental materials. As they are used in the human body, biocompatibility is an important property. Released metal ions do not always affect the human body; partners that combine with the released ions, as well as their quantity and toxicity must be considered during the assessment of metallic biomaterials (2,4-6). Metallic biomaterials implanted in the human body are exposed to severe environments, including body fluids containing chloride ions, amino acids, and various proteins, and at lower partial oxygen pressure than in air. Therefore, implanted devices made with stainless steel are reported to sometimes corrode (4).

In the present study mean cell viability of Ti grade 4 was the highest (99%), followed by Ni-625 (98%), SS-304 L (97%), and SS-321 (95%). These results are in accordance with the literature, as Ti and Ni-625 are materials widely used in orthopedic and dental implant manufacturing because they are inert, and SS-304L and SS-321 are used for manufacturing surgical devices because they are corrosion resistant. Cytotoxicity, allergy, or other biological effects of their metallic ions are considered negligible (2). Zirconium, niobium, and tantalum ions are titanium-type ions. These elements are considered components that may improve the safety of titanium alloys. On the other hand, inactive ions, such as nickel and copper, do not immediately combine with water molecules or inorganic anions; therefore, in ionic state they survive for a relatively long time. These ions have a greater probability of combining with biomolecules and creating toxicity. There are different techniques to inhibit ion release from metallic implants. Formation of an oxide layer on the surface as a passive film inhibits electrochemical reactions and, therefore, the release of ions. Nitrogen treatment is also possible and Yamamoto et al. developed nickel-free austenitic stainless steel using a nitrogen coating to avoid the toxic effect of released nickel ions (4).

Due to incomplete polymerization of polymers, polymeric extracts are much more cytotoxic than metallic extracts. The cytotoxicity of polymers is caused by the release of monomers into the extraction medium. Yoshii et al. evaluated 39 acrylates and methacrylates used in dental resin materials using the MTT assay. Additionally, the relationship between their structure and cytotoxicity was studied to predict cytotoxic levels of dental resin materials, so as to develop new low-toxicity resin materials. All the acrylates evaluated were more toxic than the corresponding methacrylates. In both the acrylates and methacrylates, a hydroxyl group appeared to increase cytotoxicity (18). Ceramics have been used successfully in total joint prostheses for a number of

Table 5. The statistical difference between the groups (P < 0.001).

One-way ANOVA followed by Bonferroni's multiple comparison test	1 MN	2 MN	3 MN
Cell Control vs Mitomycine C	***	***	***
Cell Control vs Zinc Control	***	***	ns
Cell Control vs PMMA	***	***	ns
Cell Control vs PMMA+MMA	***	***	ns
Cell Control vs PMMA+MMA+HA	***	***	ns
Cell Control vs Ti-Grade4	***	ns	ns
Cell Control vs Alloy 625	***	ns	ns
Cell Control vs Alloy 304 L	***	ns	ns
Cell Control vs Alloy 321	***	ns	ns
Mitomycine C vs Zinc Control	***	***	***
Mitomycine C vs PMMA	***	***	***
Mitomycine C vs PMMA+MMA	***	***	***
Mitomycine C vs Ti-Grade4	***	***	***
Mitomycine C vs Alloy 625	***	***	***
Mitomycine C vs Alloy 304 L	***	***	***
Mitomycine C vs Alloy 321	***	***	***
Zinc Control vs PMMA	***	ns	ns
Zinc Control vs PMMA+MMA	***	ns	ns
Zinc Control vs PMMA+MMA+HA	***	ns	ns
Zinc Control vs Ti-Grade4	***	***	ns
Zinc Control vs Alloy 625	***	***	ns
Zinc Control vs Alloy 304 L	***	***	ns
Zinc Control vs Alloy 321	***	***	ns
PMMA vs PMMA+MMA	***	***	ns
PMMA vs PMMA+MMA+HA	***	***	ns
PMMA vs Ti-Grade4	***	***	ns
PMMA vs Alloy 625	***	***	ns
PMMA vs Alloy 304 L	***	***	ns
PMMA vs Alloy 321	***	***	ns
PMMA+MMA vs PMMA+MMA+HA	***	ns	ns
PMMA+MMA vs Ti-Grade4	***	***	ns
PMMA+MMA vs Alloy 625	***	***	ns
PMMA+MMA vs Alloy 304 L	***	***	ns
PMMA+MMA vs Alloy 321	***	***	ns
PMMA+MMA+HA vs Ti-Grade4	***	***	ns
PMMA+MMA+HA vs Alloy 625	***	***	ns
PMMA+MMA+HA vs Alloy 304 L	***	***	ns
PMMA+MMA+HA vs Alloy 321	***	***	ns
Ti-Grade4 vs Alloy 625	***	ns	ns
Ti-Grade4 vs Alloy 304 L	***	ns	ns
Ti-Grade4 vs Alloy 321	***	ns	ns
Alloy 625 vs Alloy 304 L	***	ns	ns
Alloy 625 vs Alloy 321	***	ns	ns
Alloy 304 L vs Alloy 321	***	ns	ns

*** indicates significant differences between groups; P < 0.001.
ns: non-significant

years and reliance on such cements as PMMA for fixation has resulted in the contribution of these materials to the particle load in periprosthetic tissues (19). The biological disadvantages of the conventional bone cement PMMA are well established. This acrylic is known to be hazardous to bone tissue at the implantation site due to the polymerization temperature or the local effects of MMA monomer leached from the material as it cures in situ.

In the present study the PMMA, PMMA+MMA, and PMMA+MMA+HA extracts contained MMA, which we think was the source of the cytotoxicity of those materials. The literature and the results of the present study indicate that PMMA powder is the most cytotoxic polymer, followed by PMMA+MMA and PMMA+MMA+HA (18-20). Metallic material surfaces are more suitable for cell attachment than polymeric surfaces. Surface properties may affect the clinical outcome of titanium implants. Postiglione et al. (2003) investigated the effects of titanium surfaces with 3 different topographies on proliferation, differentiation, and apoptosis of human osteoblast-like cells and SaOS-2. They reported that the bone-healing response around dental implants could be affected by surface topography (23).

The presence of HA in PMMA+MMA+HA polymer induces cell attachment and cell proliferation, as bone contains HA (21,22). In the present study greater cell attachment on the PMMA+MMA+HA surface than on the PMMA+MMA surface was observed with SEM. MN frequencies for Ti grade 4, alloy 625, alloy 304L, and alloy 321 were 1.8%, 2.8%, 3.5%, and 4.2%, respectively, and there were significant differences between them (P = 0.001). We did not observe 1 MN, 2 MN, or 3MN cells in the metallic material extracts; however, the sum of 1 MN and 2 MN cells in PMMA, PMMA+MMA, and PMMA+MMA+HA was 10.8%, 8.9%, and 5.4%, respectively, all of which are relatively higher than observed in the metallic material extracts. Schweikl et al. reported MN formation frequencies for some dental composite materials that consisted of multi-acrylate monomers in V79 mouse fibroblasts. MN formation in those materials' Solitaire® and Solitaire 2® was 8% and 9% respectively (1). Surface properties of materials are directly related to cell proliferation, differentiation, and apoptosis. Ti implants that have different surface properties affect the cell phenotype and bone healing responses. Cellular response to the same agent in vivo will be very different than it is in vitro (23,24); therefore, when evaluating biocompatibility test results in vivo test results must also be taken into consideration.

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References

1. Schweikl H, Hiller KA, Bolay C, Kreissl M, Kreismann W, Nusser A et al. Cytotoxic and mutagenic effects of dental composite materials. Biomaterials 2005; 26, 1713-19.
2. Hanawa T. Metal ion release from metal implants. Materials Science and Engineering C 2004; 24, 745-52.
3. Wataha JC, Rueggeberg FA, Lapp CA, Lewis JB, Lockwood PE, Ergle JW et al. In vitro cytotoxicity of resin-containing restorative materials after aging in artificial saliva. Clin Oral Investig 1999; 3: 144-49.
4. Yamamoto A, Kohyama Y, Kuroda D, Hanawa T. Cytocompatibility evaluation of Ni-free stainless steel manufactured by nitrogen adsorption treatment. Materials Science and Engineering C 2004; 24: 737-43.
5. Spahl W, Budzikiewicz H, Geurtsen W. Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry. J Dent 1998; 26: 137-45.
6. Pelka M, Distler W, Petschelt A. Elution parameters and HPLC detection of single components from resin composite. Clin Oral Investig 1999; 3: 194-200.
7. Demirci M, Hiller KA, Bosl C, Galler K, Schmalz G, Schweikl H. The induction of oxidative stress, cytotoxicity, and genotoxicity by dental adhesives. Dental Materials 2008; 24: 362-71.
8. Souza Costa CA, Hebling J, Godoy FG, Hanks CT. In vitro cytotoxicity of five glass-ionomer cements. Biomaterials 2003; 24: 3853-58.
9. BS EN ISO 10993-1:1998, Biological Evaluation of Medical Devices, Evaluation and Testing.
10. BS EN IS0 10993-3:1998, Biological Evaluation of Medical Devices, Tests for Genotoxicity, Carcinogenicity and Reproductive Toxicity.
11. IS0 10993-5:1998, Biological Evaluation of Medical Devices, Tests for in-vitro Cytotoxicity.
12. OECD Guideline for the Testing of Chemicals Draft Proposal for a New Guideline 487, In Vitro Micronucleus Test, 2004.
13. ISO 10993-12:1998, Sample Preparation and Reference Materials
14. Jenkins N. Ed., Animal Cell Biotechnology Methods and Protocols, Cell Counting and Viability Measurements, Humana Press Inc 1999; 139.
15. Gurhan SI, Vatansever HS, Kurt FÖ, Tuglu I, Characterization of osteoblasts derived from bone marrow stromal cells in a modified cell culture system, Acta Histochemica 2006; 108: 49-57.
16. Hanawa T, Kaga M, Itoh Y, Echizenya T, Oguchi H, Ota M. Cytotoxicities of oxides, phosphates and sulphides of metals. Biomaterials 1992; 13: 20-24.
17. Volden G, Haugen HF, Skrede S, Reversible cellular damage by dimethyl sulfoxide reflected by release of marker enzymes for intracellular fractions, Archives of Dermatological Research 1980; 269: 2.
18. Lohmann CH, Dean DD, Oster GK, Casasola D, Buchhorn GH, Fink U et al. Ceramic and PMMA particles differentially affect osteoblast phenotype, Biomaterials 2002; 23: 1855-63.
19. Yoshii E, Cytotoxic effects of acrylates and methacrylates: Relationships of monomer structures and cytotoxicity, Biomed Mater Res, John Wiley & Sons, Inc, 1997; 37: 517-24.
20. Revell PA, Braden M, Freeman MAR, Review of the biological response to a novel bone cement containing poly(ethyl methacrylate) and n-butyl methacrylate, Biomaterials 1998; 19: 1579-86.
21. Rahbek O, Kold S, Bendix K, Overgaard S, Soballe K, Superior sealing effect of hydroxyapatite in porous-coated implants: experimental studies on the migration of polyethylene particles around stable and unstable implants in dogs. Acta Orthop 2005; 76: 375-85.
22. Galego N, Rozsa C, Sanchez R, Fung J, Vazquez A, Tomase JS, Characterization and application of poly(b-hydroxyalkanoates) family as composite biomaterials. Polymer Testing 2000; 19: 485-92.
23. Postiglione L, Domenico G, Ramaglia L, Montagnani S, Salzano S, Meglio F et al. Behavior of SaOS-2 cells cultured on different titanium surfaces. J Dent Res 2003; 82: 692-96.
24. Postiglione L, Domenico G, Ramaglia L, Lauro AE, Di Meglio F, Montagnani S. Different titanium surfaces modulate the bone phenotype of SaOS-2 osteoblast-like cells. Eur J Histochem 2004; 48: 213-22.