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RESEARCH ARTICLE

ASSESSMENT OF GINGIVAL FIBROBLAST CYTOTOXICITY OF RECASTED COBALT CHROMIUM DENTAL ALLOY- IN VITRO STUDY

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ABSTRACT

Statement of problem: Recasting procedure could affect the cytotoxicity of dental alloys because remaining alloy from initial castings is reused commonly with addition of new alloy.
Purpose: To evaluate the effect of recasting of cobalt chromium alloys on gingival fibroblast cytotoxicity.
Materials and Methods: Disk-shaped specimens (1 × 2 mm, n=40) of cobalt chromium alloys were prepared with 100% new alloy and 50%, 30%, and 15% once recast alloy. 10 specimens from each group were conditioned in 0.5ml of saline (0.9% NaCl) containing 3% bovine serum albumin(BSA). Cytotoxic effects were assessed on human gingival fibroblast with (MTT) colorimetric assay a 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide and neutral red uptake (NRU) assay test. Collected data was statistically analysed with one way ANOVA and Tukey's HSD multiple comparison test for each group and the effect of repeated casting of base metal alloys on gingival fibroblast cytotoxicity was evaluated.
Results: Effects of alloy type on MTT activity and NRU assay depend on the amount of recasted alloy addition (P<.001) indicating amount of recast alloy affected the cellular activity of HGF by 1-way ANOVA. Post hoc multiple comparison tests were performed to determine how casting procedures affect MTT activity NRU assay of HGF cells. Tukey's HSD test revealed that the mean MTT activity and NRU assay was significantly different for each casting procedure group (P<.001). Post hoc comparisons of the 4 casting procedures also indicated that each increase in surplus alloy amount in the alloy.
Conclusions: Recasting of cobalt chromium alloys increased cytotoxicity and decreased cell viability of human gingival fibroblasts.

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INTRODUCTION

During the human lifespan, teeth might become severely impaired by carious infection, ruptured by trauma, or lost because of extraction as a consequence of different dental diseases. A cast-metal alloy is one of the materials of choice to substitute lost tooth/teeth or crown for gravely damaged tooth. It can be used alone or in conjunction with dental ceramics when aesthetics is of prime concern. An ideal dental alloy to be used in oral cavity should be easy to melt, cast, braze, and polish. Other essential features are nominal shrinkage, high resistance to wear, high strength, and high tarnish and corrosion resistance. [1]Additionally, material should be biocompatible.[2] Dental restorations are retained in the oral surroundings directly in contact with the gingival tissue.[3] Therefore, clinicians should select a non-hazardous or the least toxic metal alloys, Later on any release of elements from these alloys can be a source to adverse tissue reactions, as shown by various animal studies.[4] Traditionally, precious metals and their alloys have been considered as ideal restorative materials. But, due to financial constraints, base metalalloys are broadly used, restricting the regular use of precious alloys in practice. Originally, base metal alloys were so low-priced that new ingots were used for every new case, but the demand for them has now lead to large increases in the rate, a point of economic concern for commercial dental laboratories.[5] For such reasons dental laboratories may combine formerly casted metal with fresh ingots of alloy to further decrease the cost. Various published articles of casting of reused dental alloys which differ broadly. They range from views that no new metal should be added to recommendations that 50% new metal may be combined with previously melted but tonsorsprues removed from castings.[6-8] Clinically local adverse tissue responses such as enhanced gingivitis and periodontit is in direct contact of dental casted alloys is believed to be associated to the

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consequence of metal cationsreleased in to the adjoining tissue.[9,10] Elements released from these alloys may subsequently interfere with many biochemical and enzymatic cellular reactions, resulting in cellnecrosis.[11,12] Types of elements that an alloy is composed of and their release into the surrounding medium govern the cytotoxic effect of the alloy.[13-21]Indeed, cytotoxicity of dental alloys has been correlated with anyalteration in their composition[22]; therefore, the relation between composition of the dental casting alloy and its cytotoxicity needs further investigations. Purpose of this study was to evaluate and compare the effect of different proportions of recasting of cobalt chromium alloys on gingival fibroblast cytotoxicity. This study tested the null hypothesis that there is no difference in the cytotoxicity of extract solution from different recasting proportions of cobalt-chromium (Co-Cr) on human gingival fibroblasts.

MATERIALS AND METHODS

Composition of the metal alloys used as indicated by manufacturers are shown in Table I. Ten specimens from each type of casting procedures (n=40) were prepared in the form of disks 1 mm in diameter and 2mm in thickness..Ten iron disks (1x2mm) were produced by the engineering workshop and placed in a plastic tray. The silicone-based duplicating material (Bego) was then poured over them into the tray and allowed to set. After the silicone material had set, it was separated from the tray, and the iron disks were removed from the silicone material, leaving 10 spaces with the same size and shape as the iron disks. These spaces were used as molds to produce the wax pattern of the casting alloy specimens. Inlay wax (Bego) was used to produce the wax pattern. Preparation of sprues, investment, burnout, and castings were done according to the manufacturer's instructions. Co-Cr (Bego-Wironit) alloys were cast with a conventional lost wax technique using phosphate bonded investment (Bego). All processes of finishing and polishing were carried out to simulate the preparation of the cast-metal alloys for clinical cases. For each alloy type, four different casting procedures were assessed according to the percentage of new (as-received) and recast alloy. The four compositions evaluated were (n=10):1) 100% new alloy group (NA-100), all the casting alloy was used for the first time2) 85% once recast alloy combined with 15% new alloy (NA-85).3) 70% once recast alloy combined with 30% new alloy (NA-70)4) 50% once recast alloy group combined with 50% new alloy (NA-50). Specimens were rinsed with distilled water and sterilized (autoclaved at 134°C for 10 minutes). Composition of alloys was stated as the weight percentage (wt%) of elements in the alloy. For extract solution process, the surface area of cylindrical specimens was determined by using the diameter and height as measured with an electronic digital caliper (), and a standard 0.63 cm^2 of the surface area of each prepared alloy specimen was exposed to the extraction medium. Extract solution was 0.5 mL of saline (0.9% NaCl) containing 3% bovine serum albumin (BSA) (Gibco Chemical Co). Every sterilized specimen was positioned in a well in a sterile 24-well polystyrenecell-culture tray (NEST Biotech Co.ltd). Four trays, containing 10specimens were used for each casting procedure type. Extraction solution was added to each well and the specimens were conditioned in this solution at 37°C for168 hours in a 5%:95% CO_2: air environment with 100% relative humidity. Amount of alloy surface area relative to the volume of the solution was 1.26 cm^2/mL, which is within the range that the International Organization for Standardization recommends for evaluating the cytotoxicity of dental biomaterials in cell culture (suggested range 0.5-6.0 cm2/mL). At the end of a 168-hour conditioning period, extract solutions were collected from the wells. As stated below, a 100-µL volume of each extract was added to a well in a cell-culture plate containing fibroblasts cultured from gingival biopsies. Specimens of human gingiva were collected with the informed consent and approval of the KLE VK I.D.S from Department of Periodontology from a healthy patient who underwent preprosthetic crown lengthening procedures. A 3 × 3 × 3 mm biopsy of marginal gingiva was taken from the anterior buccal, mesial, and distal aspect of the teeth that were being surgically treated. Teeth adjacent to the site of tissue removal exhibited no periodontal or endodontic infection. The patient had no restorative material adjacent to the gingivectomy site, and no crowns or fixed prostheses in this area. The biopsies obtained during surgery were placed in a 1.5 mL Eppendorf tube containing Dulbecco's modified Eagle medium (DMEM) (Gibco Chemical Co) augmented with 15% fetal bovine serum 100 U/mL in penicillin G, 100 µg/mL streptomycin, and 50 ng/mL amphotericin B (all materials from Gibco Chemical Co), and were instantly transported to the laboratory for the cell culture procedure. Gingival specimen was transported to a tissue-culture dish and separated into small pieces (approximately 1 ×1 × 1 mm). These explants were then transferred to tissue-culture flasks, and culturing was done in DMEM supplemented with 15% foetal bovine serum (FBS)(Gibco Chemical Co), 100 U/mL penicillin G,100 µg/mL streptomycin, and 50 ng/mL amphotericin B. The tissues were initially incubated in 75 cm2 flasks at 37°C in a humidified 5%:95% CO_2 air environment. When the cells growing from the explants became confluent, they were treated with 0.2% trypsin solution in phosphate-buffered saline (Himedia laboratories Pvt ltd) and transferred to new tissue-culture flasks for secondary culture. This procedure was done every 5 days and the culture medium was renewed twice a week. Cells were passaged 4 to 7 times.

Cytotoxicity was measured in terms of cellular viability as concluded by mitochondrial enzyme activity of HGF cells. Before testing supernatant from each flask was collected and then centrifuged at 12,000 gm Cultured cells were pooled and counted in a haemocytometer. Appropriate dilutions were made with DMEM + 15% FBS, and the cells were plated out at a density of 12,500 cells/cm2. Cells were transferred to two 96-well cell-culture platestray (NEST Biotech Co. ltd)and incubated for 24 hours. To avoid problems with evaporation, the outmost rows of wells on each plate were not used. Thirty wells at the centre of the plates were used as test wells. Two columns of each plate (24 wells) were used as control wells (cells not exposed to extract).Six wells (1 column to the right of the control wells on the right side) were used as blanks for spectrophotometry analysis. After the 24-hour incubation period, as noted above, 100 µL of each extract solution obtained from each specimen (n=40) was added to a test well in the culture plates. Plates were then incubated for another 72 hours. Effects of different extracts were assessed based on the amount of mitochondrial activity after incubation.

In metabolically active cells, yellow tetrazolium salt (MTT) is reduced to form purple for mazan crystals, which can be dissolved by adding detergent. After dissolution, colour quantification can be done spectrophotometrically. A linear relationship between the number of cells and absorbance is established for each cell type. This allows accurate, straightforward quantification of changes in cell proliferation,

which can be expressed as mitochondrial dehydrogenase activity. For this study, instantly before use a 5-mg/mL solution of MTT(3-[4.5-dimethylthiazol-2yl]-2.5-diphenyltetrazolium bromide) was prepared in warm phosphate-buffered saline. A 20-µL aliquot of MTT solution (Himedia laboratories Pvt ltd) was added to each test and control well in the 2 plates. Plates were agitated for 10 minutes on a plate shaker and were then incubated for another 4 hours in a CO_2incubator. All test and control wells in each plate were then vacuum aspirated, and 200 µL of dimethylsulfoxide (DMSO) (Fisher Scientific) was added to each well. Plates were agitated to promote dissolution of the formazan crystals. Absorbance of the material from each well at 630 nm was measured in a spectrophotometer by using DMSO as the blank. Mean absorbance values of the control cells (material from untreated wells) were taken to represent 100% cell viability. From absorbance values, the mean mitochondrial dehydrogenase activity of each subgroup for each alloy was calculated as a percentage of the control activity.

For neutral red assay test100ml of Neutral Red Solution () was added to each well and kept for 3 hours in incubator. At the end of the incubation period, the medium was carefully removed and the cells quickly rinsed with Neutral Red Assay Fixative [N-4270]. Extended fixation times can result in leaching of the dye into the fixative solution. Alternatively the cells were washed in an osmotically balanced saline solution HBSS. The fixative was removed and the incorporated dye was then solubilized in a volume of Neutral Red Assay Solubilisation Solution [N-4395] equal to the original volume of culture medium. The cultures was allowed to stand for 10 minutes at room temperature. Spectrophotometric ally absorbance measured at a wavelength of 630 nm. Descriptive statistics for MTT test and NRU assay were expressed as mean standard deviation(SD). One-way ANOVA was used to determine interactions for 4 casting procedures and their effects on MTT activity. Tukey's Honestly Significant Difference (HSD) test was used for post hoc multiple comparisons.

RESULTS

The aim of this study was to examine the effect of adding various amounts of surplus alloy to Co Cr, on the metabolic activity of HGF cells. Table II and III summarizes the absorbance readings at 630 nm (MTT Assay and neutral red assay) of control and the four casting procedure groups. One-way ANOVA revealed significant differences between the groups of casting procedures (P<.001). Results of the One-way ANOVA, revealed that the effects of alloy on MTT activity and neutral red uptake assay depend on the amount of recast alloy addition (*P*<.001) indicating amount of recast alloy affected the cellular activity of HGF. Post hoc multiple comparison tests were performed to determine how casting procedures affect MTT activity of HGF cells. Tukey's HSD test revealed that the mean MTT activity was significantly different for each casting procedure group (*P*<.001). Post hoc comparisons of the 4 casting procedures also indicated that each increase in surplus alloy amount in the alloy composition resulted in significantly lower MTT activity levels (*P*<.001). Significant differences in the metabolic activity of HGF cells and controls are shown in Figure 1. Post hoc multiple comparison tests were performed to determine how casting procedures affect the ability of HGF cells to incorporate and bind the supravital dye neutral red. Tukey's HSD test revealed that the mean NRU assay was significantly different for each casting procedure group (*P*<.001). Post hoc comparisons of the 4 casting procedures also indicated that each increase in surplus alloy amount in the alloy composition resulted in significantly decreased uptake (*P*<.001). Significant differences in the metabolic activity of HGF cells and controls are shown in Figure 2.

Table 1. Composition according to weight percentage by manufacturer (Bego-Wironit)

Cobalt (Co)	62.5%
Chromium(Cr)	29.53%
Molybdenum(Mo)	5%
Silicone(Si)	<2%
Manganese(Mn)	<2%
Ferrum(Fe)	<2%
Tantalum(Ta)	<2%
Carbon(C)	<2%
Nitrogen(N)	<2%

Table 2. Absorbance Readings at 630 nm (MTT Assay) of Cells Cultured in the Presence and Absence (Control) of the Alloy Specimens

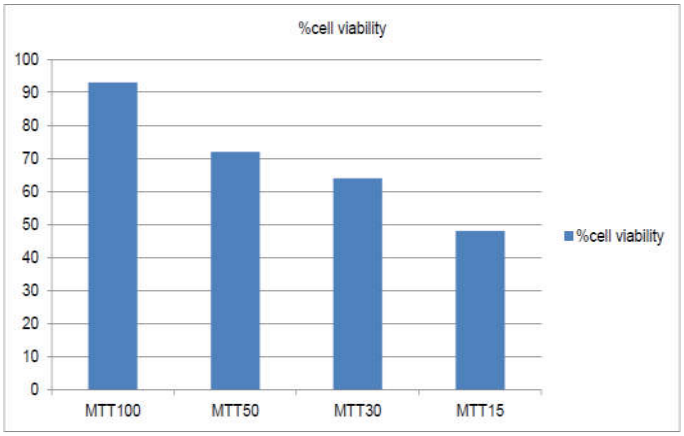
Test Group	Viable Cells Mean SEM
Control	.632+.150
100% new	.908+.159
50% reused + 50% new	1.285+1.306
70% reused+30% new	.549+.108
85% reused + 15% new	.571+.087

F= 2.746 *P*<.001

Table 3. Absorbance Readings at 630 nm (Neutral Red Uptake Assay) of Cells Cultured in the Presence and Absence (Control) of the Alloy Specimens

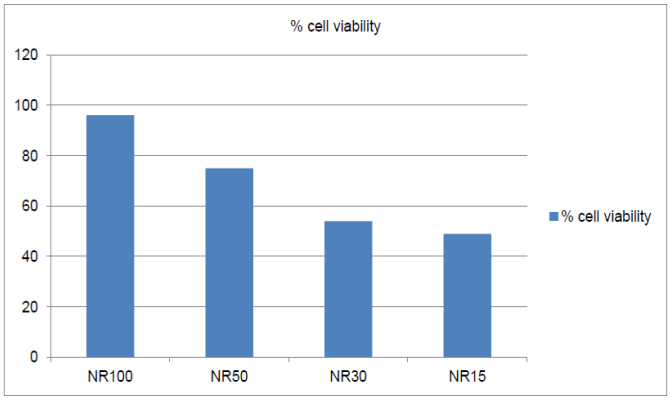
Test Group	Viable Cells Mean^{+}. SEM
Control	.111+.008
100% new	.107+.008
50% reused + 50% new	.095+.024
70% reused+30% new	.161+.019
85% reused + 15% new	.221+.081

F= 26.513 *P*<.001



MTT100= 100% alloy
MTT50=50:50% alloy
MTT30=70:30% alloy
MTT50=85:15% alloy

Figure 1. Percentage of cell viability of HGF cells against casting procedures by MTT colorimetric assay



NR100= 100% alloy
NR50=50:50% alloy
NR30=70:30% alloy
NR50=85:15% alloy

Figure 2. Percentage of cell viability of HGF cells against casting procedures by

DISCUSSION

Because of better mechanical properties and decreased cost, Co-Cr base metal alloys have gradually substituted gold alloys in the manufacture of fixed metal ceramic restorations. Presently, wide number of commercial dental laboratoriesare using recast alloy (used once or more times) eitheralone or by combining fresh metal ingots for fabricating dental prosthesis, considering only cost factor of the alloy, with no regard to manufacturers guidelines or changes in its properties on reusage. These casting procedures change chemical composition, microstructure, physical properties, and cytotoxicity of alloys.[23,3,24] Therefore, it becomes vital to determine if there is any elevation in cytotoxicity of Co− Cr alloys in the reused state when compared to its first time use. Results of this study demonstrated that extract solutions from all 4 casting procedures were more cytotoxic than the controlcultures. Based on the results of statistical analysis, the null hypothesisis rejected because all 4 casting procedures significantly affected HGF cell viability. Biological acceptability of dental casting alloys was assessed with in vitro cytotoxicity testing.[25] International standards compiled as ISO 10993 mention in vitro cellulartoxicity testing on established celllines.[26] For this purpose permanent mouse fibroblasts(L-929, 3T3) or human epithelial cells (HeLa) are being used.But, standards recommend that primary cultures from biopsies of target tissues such as gingival or pulp fibroblasts could also be selected. Watahaet *al*[27] evaluated the responses of 4cell lines (Balb/c 3T3, L929, ROS17/2.8, and WI-38) to 14 metal ions. Restorations frequently extend into the gingival sulcus and gingival epithelial cells are the firstcells to come into interaction with them. Studies assessing effects of subgingival restorations on periodontal health have revealed that margins beneath the gingiva considerably compromisegingival health.[1] Therefore, cytotoxictesting of dental alloys on epithelialcells would better associate to in vivoconditions. Though, oral epithelialcells used for this purpose are derived from epidermoid carcinoma; primary cultures have a more standard phenotype and associate to in vivo response more precisely.[1] In the present study, HGF were selected because such cellsare exposed to dental restorations when ulceration of the epithelium follows after gingival inflammation. Primary gingival fibroblast cultures were used instead of cloned cell lines because of the ability of these cells to retain many of the distinguished characteristics they display in vivo.

Various studies have used diverse conditioning solutions, to simulate the oral environment invitro.[28-30] Saline with BSA was used as conditioning solution for this study. This choice was based on earlier findings that solutions containing protein cause augmented alloy corrosion and that corrosion is a necessary for the occurrence of biologiceffects.[30] Dental alloys remain in the intraoral environment for many years, so it is important to know what occurs with extended exposure. In present study, conditioning of castalloys and application of extracts to gingival fibroblasts were done by using the indirect contact method.[31]With this technique, it is possible to evaluate effects of longer periods of fibroblast exposure to the conditioning media. According to Wataha *et al*[31] the indirect method is the most appropriate system for this type of assessment. George Fotakis[32]conducted a study in which his aim was to compare four in vitro cytotoxicity assays and determine their ability to detect early cytotoxic events. In conclusion neutral red and the MTT assay showed the maximum sensitivity in detecting cytotoxic events compared to the LDH leakage and the protein assay. For evaluation of cell death due to corrosion products released from test alloy specimens, MTT colorimetric assay and neutral red assay was used. Effects of different extracts were assessed based on the amount of mitochondrial activity after incubation. A linear relationship between the number of cells and absorbance is established for each cell type. Amount of for mazan formed is directly proportional to the number of viable cells in the sample. All the four groups of alloys showed statistically significant decline in viable cell counts when compared to control. The greatest decline in viable cell counts was observed for 70% and 85% reused alloy groups.

Neutral red uptake assay provides a quantitative estimation of number of viable cell in culture. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in lysosomes. All four groups of alloys showed statistically significant decline in viable cell counts when compared to control. However cytotoxicity for 100% new metal alloy and 50% reused alloys was not significant when compared to control group for both the assays. Results of One-way ANOVA, revealed that the effects of alloy on MTT activity and neutral red uptake assay depend on amount of recast alloy addition (P<.001) indicating amount of recast alloy affected the cellular activity of HGF. Decline in the percentage of recasted alloy increases cellular activity and decreases cytotoxic affect. Imirzalioglu *et al*[1] reported similar results to the current study that adding recast alloy negatively influenced the cellular activity of all tested alloys (P<.001). Studies have demonstrated that higher chromium content in base metal alloys results in higher corrosion resistance.[19,33,34]In a study conducted by Imirzalioglu *et al*[1], it was found thatCo-Cr alloy which had a higher chromium content featured less chromium than cobalt release and had fewer severe cytotoxic effects than Ni-Cr alloy subgroups. Ameer *et al*[35] also stated that Co-Cr alloys exhibit better corrosion behaviour than Ni-Cr alloys even after repeated use. Probable reason might be that when in contact with oxygen or moist media, chromium forms a passive oxide layer tha tinterferes with corrosion circuit. Al-Hiyasat and Darmani[3] reported more cobalt release from Co-Cr alloy (Wirobond C) than nickel release from Ni-Cr alloys (RemaniumCS and Wiron 99). They also stated that cytotoxicity of this alloy could be a result of increased

cobaltrelease, which is more toxic than nickel. Results of the current study indicate that for each casting procedure Co-Cr alloys were more cytotoxic than control group. It is now well known that metal ions from various dental restorations are released and penetrate enamel, dentin and gingiva.[36-39]causing local symptoms.[40]

Conclusion

Adding recast alloy negatively influenced the cellular activity of all tested alloys (P<.001).Considering the elevated cytotoxicity of Co−Cr base metal alloys in their reused state, the practice of reusing these alloys in dental laboratories for financial reasons should be discouraged. However, further studies can be directed to evaluate and compare the effect of different proportions of recasting of cobalt chromium alloys on gingival fibroblast cytotoxicity, in order to consolidate the results of this study.

REFERENCES

1. Pervin Imirzalioglu, Influence of recasting different types of dental alloys on gingival fibroblast cytotoxicity. J Prosthet Dent 2012;107:24-33
2. Williams D. Revisiting the definition of biocompatibility, Medical Device Technology 2003;14(8).
3. Al-Hiyasat AS, Bashabsheh OM, Darmani H. An investigation of the cytotoxic effects of dental casting alloys. Int J Prosthodont 2003;16:8-12
4. Reuling N, Wisser W, Jung A, Denschlag H. Release and detection of dental corrosion products in vivo: Development of an experimental model in rabbits. J Biomed Mater Res 1990;24:979–991
5. T.S. Chandra, N.S. Kumar, B.K. Kumari,Evaluating Cytotoxicity of Recycled Ni-Cr Dental Casting Alloys – An In Vitro Study Trends Biomater. Artif. Organs. 25, 51 (2011).
6. Anusavice KJ. Phillips' science of dental materials. Philadelphia: W.B. Saunders Co;2003. p. 457-63.
7. Powers JM, Sakagushi RL. Craig's Restorative dental materials. 12th ed. St Louis: Mosby; 2006. p. 514-42.
8. Baum L, Phillips RW, Lund MR. Textbook of operative dentistry. 3rd ed. Philadelphia:WB Saunders; 1994. p. 470-93.
9. Wirz J. Schädigung des Parodontes durch zahnärztlich Werkstoffe.Zahnarztl Welt Zahnarztl Rundschau 1993;102:146–162.
10. . Wirz J, Haefeli D, Schmidli F. Aufbrennlegierungen im Korrosionstest. Die Quintessenz 1993;44:75–84.
11. Bumgardner JD, Lucas LC. Cellular response to metallic ions released from Ni-Cr dental alloys. J Dent Res 1995;74:1521–1527.
12. Schedle A, Samorapoompichit P, Rausch-Fan XH, *et al*. Response of L-929 fibroblasts, human gingival fibroblasts, and tissue mast cells to various metal cations. J Dent Res 1995;74:1513–1520.
13. Craig RG, Hanks CT. Reaction of fibroblasts to various dental casting alloys. J Oral Pathol 1988;17:341–347.
14. Wataha JC, Lockwood PE. Release of elements from dental casting alloys into cell culture medium over 10 months. Dent Mater 1998;14:158–163.
15. Craig RG, Hanks CT. Cytotoxicity of experimental casting alloys evaluated by cell culture tests. J Dent Res 1990;69:1539–1542.
16. Grill V, Sandrucci MA, Di Lenarda R, *et al*. In vitro evaluation of biocompatibility of dental alloys: Fibronectin expression patterns and relationships to cellular proliferation rates. Quintessence Int 2000;31:741–747.
17. Bumgardner JD, Lucas LC. Surface analysis of Ni-Cr dental alloys.Dent Mater 1993;9:252–259.
18. Brune D. Mechanism and kinetics of metal release from dental alloys. Int Endod J 1988;21:135–142.
19. Kaga M, Seale NS, Hanawa T, Ferracane JL, Waite DE, OkabeT. Cytotoxicity of amalgam alloys and their elements and phases.Dent Mater 1991;7:68–72.
20. Wataha JC, Craig RG, Hanks CT. Element release and cytotoxicity of Pd-Cu binary alloys. Int J Prosthodont 1995;8:228–232.
21. Goehlich V, Marek M. Corrosion behavior of Pd-Cu and Pd-Co alloys in synthetic saliva. Dent Mater 1990;6:103–110.
22. Sjögren G, Sletten G, Dahl JE. Cytotoxicity of dental alloys,metals, and ceramics assessed by millipore filter, agar overlay,and MTT tests. J Prosthet Dent 2000;84:229–236.
23. Hong JM, Razzoog ME, Lang BR. The effect of recasting on the oxidation layer of a palladium-silver porcelain alloy. J Prosthet Dent 1988;59:420-5.
24. Stamenković D, Čairović A, Čolić M, Rudolf R, Radović K, Đorđević I. The effect of repeated casting on the biocompatibility of a dental gold alloy. Acta Veterinaria 2009;59:641-652.
25. Schmalz G, Arenholt-Bindsle, D. Biocompatibility of dental materials. Heidelberg: Springer Berlin, 2009. p.396.
26. International Organization for Standardization. ISO 10993-5:2009. Biological Evaluation of Medical devices, Part 5 - Tests for invitro cytotoxicity. Geneva: ISO; 2009. Available at: http://www.iso.org/iso/store.htm
27. Wataha JC, Hanks CT, Sun Z. Effect of cell line on in vitro metal ion cytotoxicity. Dent Mater 1994;10:156-61.
28. Craig RG, Hanks CT. Reaction of fibroblasts to various dental casting alloys. J Oral Pathol 1988;17:341-7.
29. Wataha JC, Lockwood PE, Khajotia SS,Turner R. Effect of pH on element release from dental casting alloys. J Prosthet Dent1998;80:691-8.
30. Wataha JC, Nelson SK, Lockwood PE. Elemental release from dental casting alloys into biological media with and without protein. Dent Mater 2002;8:396-406.
31. Wataha JC, Lockwood PE, Nelson SK,Bouillaguet S. Long-term cytotoxicity of dental casting alloys. Int J Prosthodont 1999;12:242-8.
32. George Fotakis, John A. Timbrell. In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicology Letters 160 (2006) 171–177 173
33. Geis-Gerstorfer J, Sauer KH, Passler K. Ion release from Ni-Cr-Mo and Co-Cr-Mo casting alloys J. Prosthodont 1991;4:152-8.
34. Jones SB, Taylor RL, Colligon JS, Johnson D. Effect of element concentration on nickel release from dental alloys using a novel ion beam method. Dent Mater 2010;26:249-56.
35. Ameer MA, Khamis E, Al-Motlaq M. Electrochemical behaviour of recasting Ni-Cr and Co-Cr non-precious dental alloys, Corros Sci 2004;46:2825-36.
36. Arvidson K, Wroelewski R. Migration of metallic ions from screw posts into dentin and surrounding tissues. Seand J Dent Res 1978; 86:200-5.

37. Bergenholtz A, Hedegard E,Sremark R Studies of the transport of metal ions from gold inlays into environmental tissues. Aeta Odontol Scand 1965: 23: 135^6.
38. Soremark R, Ingels O , P L E T T H , Samsah K. Influence of some dental restorations on the concentrations of inorganic constitoents of the teeth. Acta Odontol Scand 1962; 20: 215-24.
39. Soremark R, Wing K, Olsson K, Goldin J. Penetration of metallic ions from restorations into teeth. J Prosthet Dent 1968; 20: 531-40.
40. Lundstrom IMC. Allergy and corrosion of dental materials in patients with oral lichen planus. Int J Oral Surg 1984; 13: 16-24.

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