The Effect of Metal Ions on Viability of L 929 Mouse Fibroblasts

L 929 Fare Fibroblastlarının Canlılığı Üzerine Metal İyonlarının Etkisi

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ABSTRACT Objective: In dentistry, several base metal casting alloys are used for restorative materials. When selecting a dental casting alloy for clinical situation, factors such as physical properties and biocompatibility of the alloy, are the most important. Clinical observation of local tissue reactions of the dental casting alloys is thought to be related to the effect of metal ions released into the surrounding tissues. Biocompatibility of alloys or released elements from them is evaluated in vitro between living tissues and alloys or metal ions by considering different metabolic criterias of the culture cell. In our study, the toxic effect of diluted and concentrated solutions of metal ions (Ni, Cr, Co, Ag, Cu, Zn, Mo, Be) were evaluated by measuring the cell viability using L 929 mouse fibroblasts. **Material and Methods:** L 929 mouse fibroblast (80.000/mL) and DMEM solution were placed in the cell culture wells. The diluted and concentrated metal ion solutions (100 µL) were placed separately in cell culture wells and left for 72 hours. The cell culture wells without metalic ion under the same conditions are used as control groups. After exposure time, the medium was removed from the cell culture wells. The cells were washed, fixed and stained by cryrstal violet. Optical density (absorbance) is measured spectrophotometrically at 492 nm. Toxicity of metal ions was determined directly by measuring the optic density of stained cells. Results were evaluated by using non parametric statistical analysis. **Results:** The diluted and concentrated metal ion solutions which incontact with L 929 mouse fibroblasts were demonstrated a significant toxicity as they were compared to control groups. The differences were statistically significant ($p< 0.05$). Low and high concentrated solutions of Be, Ag, Cu had the highest toxicity followed by Ni, Cr, Co, Zn. The lowest toxicity level was observed in Mo. **Conclusion:** It has an deleterious toxic effect even at low concentration metal ion is incontact with cell.

Key Words: Cell survival ; dental materials

ÖZET Amaç: Diş hekimliğinde temel metal alaşımları restoratif materyal olarak kullanılmaktadır. Klinikte dental döküm alaşımı seçileceği zaman, alaşımın fiziksel özelliği ve biyolojik uyumluluğu gibi faktörler çok önemlidir. Dental döküm alaşımlarının lokal doku reaksiyonlarının klinik bulgularının, çevre dokularda açığa çıkan metal iyonlarının etkisi ile olduğu düşünülür. Alaşımların biyolojik uyumluluğu veya element salınımları canlı doku ile alaşımlar veya metal iyonları temasa getirilerek, kültür hücrelerin farklı metabolik kriterleri üzerinde in vitro olarak değerlendirilmektedir. Çalışmamızda alaşımlardan salınan seyreltik ve derişik metal iyon solüsyonlarının (Ni, Cr, Co, Ag, Cu, Zn, Mo, Be) L 929 fare fibroblastlarının canlılığı üzerindeki toksik etkileri hücre kültürünün canlılığı üzerinde incelenmiştir. **Gereç ve Yöntemler:** L929 fare fibroblastları (80.000/mL) ve DMEM solüsyonu hücre kültürü tabletlerine her bir solüsyon için ayrı ayrı yerleştirildi. Daha sonra seyreltik ve derişik metal iyon solüsyonları (100 µL) ilave edilerek 72 saat temasta bırakıldı. Aynı şartlar altında metal iyonsuz hücre kültürleri kontrol grubu olarak kullanıldı. Beklenen süre sonunda sıvı ortam uzaklaştırılarak, kalan hücreler yıkanmış, fikse edilmiş kristal viyole ile boyandı. Optik yoğunluk (absorbsiyon) 492 nm dalda boyunda spektrofotometrik olarak ölçüldü. Metal iyonlarının toksisitesi direkt olarak boyanmış hücrelerin optik yoğunluğu olarak tespit edildi. **Bulgular:** Seyreltik ve derişik metal iyon solüsyonları ile temasta olan fare fibroblastlarının canlılığı, kontrol grubu ile karşılaştırıldığında önemli olarak azalmıştır ($p< 0.05$). Seyreltik ve derişik Be, Ag, Cu iyon solüsyonlarının hücre canlılığı üzerindeki etkileri Ni, Cr, Co, Zn ile gözlenen sonuçlardan daha toksiktir. En düşük toksisite Mo iyonu ile gözlenmiştir. **Sonuç:** Metal iyonları tek başlarına düşük dozlarda olsalar bile hücre canlılığı üzerine önemli etki yaparlar.

Anahtar Kelimeler: Hücre canlılığı; dental materyaller

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Base metal dental alloys (nickel-based alloy) have been used in dentistry since 1930. These alloys are usually multiphase and contain several metal ions that are known to be biologically active.[1,2]

The use of base metal alloys in living tissues neccessitates to evaluate them biologically. The release of metalic elements from dental alloys is potential health problem to the dental patient. The most important factor in determination of the biologic safety is corrosion. Intraoral corrosion is a very complex process and dependent on compositions and metallurgical state, combinations within a construction, surface conditions, mechanical aspects of function and local and systemic host environment. Composition and corrosion aspects, as well as the toxicology of the single metals and alloys, are especially prone to evoke adverse reactions.[3-5] Elemental release from alloys has also been studied extensively in different in vitro conditions.

In sufficient concentrations of metal alloys or metal ions are known to cause toxic, inflamatory, allergic or mutogenic reactions. Cases of oral tissue reactions in contact with dental cast alloys have been reported in literature.[6-8] Various investigators have used alloys or metal salt solutions to evaluate morphology, viability, proliferation, protein production and the effect on bacterial growth.[9-11] The metal salt solution showed slightly higher toxicity values than the medium extract for a range of different dental alloys.[12] However, these data cannot be extrapolated to metal or alloys and to their use in the mouth. Metals like nickel or copper being released from certain dental cast alloys were thought to be the (toxic) cause of reaction such as gingival inflamation.[13]

Research has shown that the in vitro cytotoxicity of dental casting alloys may correlate with the release of elements from the alloys. In vitro biocompatibility tests were developed to stimule and predict biological reactions to materials when placed into or on tissues in the body.[9,10,14] These tests have evaluated the cellular response to dental casting alloys, their constituents and leachable components. An important consequence of element release from dental alloys is cytotoxicity in the adjacent tissues.

These cell culture tests have evaluated the cellular response morphology, viability, proliferation, protein production, hemolysis of cells of dental casting alloy as used alloys and/or metal salt solutions.[9] However, the more sensitive cell proliferation test was required to detect effects of the alloys on the cells.

The aim of this study is to evaluate in vitro toxic effect of low and high concentrations of Ni, Cr, Co, Ag, Cu, Zn, Mo, Be ions that are known to be released from dental alloys using in vitro cell culture on L 929 mouse fibroblast at 72 hours. The hypothesis was that the effect of these metal ions on cell activity depends on their concentration and activity.

MATERIAL AND METHODS

PREPARATION OF METAL ION SOLUTIONS

The metal ions, their sources and the ranges of concentrations are shown in Table 1. These lowest and highest concentration ranges of metal ions were selected because they are assumed to be released from dental casting metal alloys into the oral environment.[5,6,8]

The diluted and concentrated metal ion solutions were prepared with 1000 mL distilled water (Riedel-de Haen, Sigma-Aldrich GmbH Seelze, Germany)

TABLE 1: Metal ion concentrations and sources.

Metal ions	Solutions	Concentrations (µmol/ L)
Ni^{+2}	$NiCl_2.6H_2O$	10-500
Cr^{+3}	$CrCl_3.6H_2O$	10-2000
Co^{+2}	$CoCl_2.6H_2O$	10-200
Ag^{+1}	Ag_2S0_4	1-20
Cu^{+2}	$CuCl_2.2H_2O$	10-500
Zn^{+2}	$ZnCl_2.6H_2O$	1-50
Mo^{+5}	$MoCl_5$	10-1000
Be^{+2}	$BeSO_4.4H_2O$	10-500

CELL CULTURE PREPARATION

DMEM medium (Dulbecco's Modified Eagle's Medium-Ham's F-12 pH 7.4; Sigma) containing 10% Fetal Bovine Serum (FBS) and christalize penicilin G pothasium (100 mL/2 million IU), christalize streptomycin sulphate (100 mL/2 g) were placed in 96-well dishes well (Sigma-Aldrich Corp., St. Louis, MO, USA), 37°C in 5% CO_2 and 97% air humidified atmosphere. Then, 80.000 cells/mL L 929 mouse fibroblasts (NCTC clone 929, ATCC CCL 1, American Type Culture Collection, Rockville Md.) were added to these culture wells and incubated for 24 hours before addition of the metal ion solutions.

CELL VIABILITY TESTS

After incubation, the diluted metal ion solutions were separately added (100 µL per well) in the cell culture wells for 72 hours for Ni, Cr, Co, Ag, Cu, Zn, Mo, Be. These procedure was repeated for concentrated metal ions. The cell culture wells without metalic ions under the same conditions are used as control groups.

After exposure time, metal ion solutions and DMEM were removed from wells. Then cells were washed with Dulbecco's phosphate-buffered saline (PBS) and fixed with 10% formaldehyde and afterwards the cells were washed again, dried and stained with crystal violet (Merck, Germany) for 20 min at 37°C. After staining, they were washed with PBS for three times. Ethyl alcohol 70% 100 µL was added for 5 min at 37°C. The same procedure was repeated for all cells including the control groups.

The viability of the cells exposed to diluted and concentrated metal ions were determined by measuring the absorbance of crystal violet at 492 nm wave length spectro-photometer (ELISA; Multiscan, Helsinki, Finland). The cell viability was analized directly by measuring optical density of the stained cells. Each experiment was repeated three times. Results were evaluated by non-parametric statistical analyses. Wilcoxon and Kruskal-Wallis analysis of variance was used to analyze the data for cytotoxicity of diluted and concentrated metal ions. Follow up comparisons between the groups were carried out using multiple comparison test.

RESULT

The effects of different metal ions and different concentrations on cell viability are shown in Figure 1.

Significant decrease were observed on viability of L 929 mouse fibroblasts that were in contact with diluted and concentrated metal ion solutions as they were compared to the control groups. The differences between low and high concentrations of metal ion solutions were statistically significant ($p< 0.05$). This may suggest that high concentrated range accelerates the cell necrosis.

The effect of the differences between metal ions on cell viability in diluted solutions are found to be statistically insignificant comparing Ni with Cr, Cu and Zn; Cr with Cu and Zn; Ag with Be; Cu with Zn. However, the differences between the other elements are statistically significant ($p< 0.05$) (Table 2).

The effect of the differences between metal ions on cell viability in concentrated solutions are found to be statistically insignificant comparing Ni with Co, Ag, Cu, Be and Zn; Cr with Mo and Zn; Co with Ag, Cu and Be; Ag with Cu and Be; Cu with Be and Zn. However, the differences between the other elements are statistically significant ($p< 0.05$) (Table 3). In our results, Be, Ag and Cu had the highest toxicity followed by Ni, Cr, Co, Zn in the diluted and concentrated solutions. The lowest toxicity level was observed in Mo.

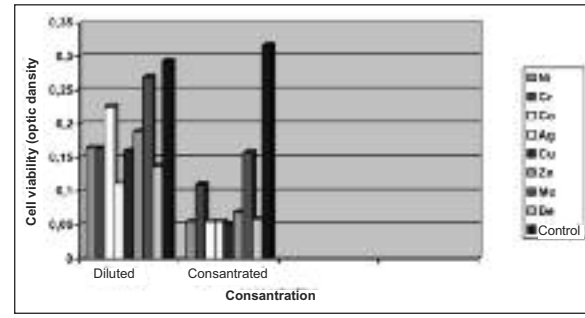

FIGURE 1: The effects of diluted and concentrated metal ions on cell viability.

TABLE 2: A comparison of diluted metal ions. Values are median, maxima and minima from triplicates, the data are expressed as percentage of untreated control cultures. The differences of cell viability was significantly different ($p < 0.05$).

Metal ions	Median	(Min-Max)	*
Ni	0.164	(0.146-0.198)	A
Cr	0.164	(0.116-0.199)	AB
Co	0.226	(0.143-0.240)	C
Ag	0.112	(0.048-0.163)	CD
Cu	0.160	(0.155-0.171)	A
Zn	0.189	(0.107-0.233)	AB
Mo	0.270	(0.228-0.297)	E
Be	0.137	(0.085-0.148)	D
Control	0.293	(0.275-0.316)	F

*Difference between mean values that are showed with different letters is statistically significant.

TABLE 3: A comparison of concentrated metal ions. values are median, maxima and minima from triplicates, the data are expressed as percentage of untreated control cultures. The differences of cell viability was significantly different ($p < 0.05$).

Metal ions	Median	(Min- Max)	*
Ni	0.055	(0.050-0.065)	A
Cr	0.110	(0.103-0.11 5)	B
Co	0.055	(0.046-0.059)	AC
Ag	0.055	(0.047-0.066)	AC
Cu	0.052	(0.047-0.061)	AC
Zn	0.069	(0.052-0.092)	D
Mo	0.157	(0.150-0.163)	B
Be	0.059	(0.031-0.085)	AC
Control	0.316	(0.245-0.369)	E

*Difference between mean values that are showed with different letters is statistically significant.

DISCUSSION

Biological and toxicologic properties of dental materials are important in relation their clinical usage. Usage of different restorative materials in dentistry requires a compatible biological relationship between materials and living tissues. Corrosion shows the biological stability of dental alloys. Corrosion usually occurs when metallic materials are exposed to body liquids. Several in vitro studies have shown that metal ions released by dental casting alloys due to corrosion may lead to local and sistemic toxicity, allergy, mutagenesis or carsinogenesis in living tissues.[4,5,15] The first step to approach dental cast alloy toxicity is to analyze the toxic potential of metal ions; e.g. in cell culture systems. Data from such experiments are dependent upon the cell culture conditions chosen; e.g the cell line, cell culture medium, incubation time.[3,16-18]

In vitro cytotocxicity test are necessary screening step in the testing of new materials used in human systems, especially tissue and cell cultures, are used in these types of studies**.** These in vitro experiments are done within 3 to 7 days.[5,6,8] Keeping the alloys and cells in biological medium more than one month causes contaminaton with microorganisms and decreases in medium component. Due to the limitations about cultured cells, a vast number of experiments are performed in less than 168 hours. The interval used to measure the cytotoxicity of dental alloys can have a significant effect on the results. Thus, this interval for using these tests to measure the cytotoxicity of alloys should be selected.[11,12,19]

In our study, it is hypothesized that metal ion concentration is important factor affecting the relationship between material and living tissue. For this reason, the effect of low and high concentrations of metal ions on viability of L 929 mouse fibroblasts was observed after 72 hours. These elements were Ni, Cr, Co, Ag, Cu, Zn, Mo, Be that known to be released from base metal alloys.

In vitro experiments include studies with metal samples as well as metal particles, metal oxides and metal ion solutions.[13,16] A specific concentration responses about a cytotoxic effects. On the other hand, the elements which damage living tissue even at low concentrations are accepted as cytotoxic elements comparing to the other elements.[20-23]

Craig and Hanks[17] stated that Ni ion that had an effect on the morphologies of cell organelles could change some amino systems. They suggested that Ni was more active in cell life than Cr. In current study, both concentrated and diluted Ni ion solutions were found to be statistically ($p < 0.05$) more active in cell viability than Cr after 72 hours.

Previous investigations have reported that the greater cytotoxic effect of the Ni-Cr alloys in comparison to the high-noble alloy was most probably related to the amount of their released elements. These findings agree with other reports, which show that Ni-Cr alloys and their products adversely affect the activity of cells.[5,7] The cytotoxicity of dental casting alloys depends on the composition of the alloys. The biological liabilities may be related to both the total mass released and the elements, such as Ni, which tend to be released from these types of alloys.[10,12,22]

Wataha and et al[21] investigated mitochondrial activity and total cell protein level using MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide succinate] assay on human macrophages with Ni, Ag, Cu and Hg and some ions at low concentrations. The ions affected both of two metabolic activities significantly. Furthermore, it was concluded that each ion had its own specific effect mechanism. We obtained similar results in this study using L 929 fibroblasts. In our study, viability of L929 mouse fibroblasts that were incontact with diluted and concentrated metal ion solutions demonstrated a significant decrease as they were compared to control group. The differences between low and high concentration were statistically significant ($p< 0.05$). Studies by Wataha et al. have shown that various metal cations cause cytopathogenic effects in murine 3T3 fibroblasts and that degree of cell damage varies depending on the concentration and type of metal cation. These studies have shown that distinct metal cations may be released from dental alloys in vitro, although, usually, toxic concentrations may not be reached.[24-29]

Wataha and et al[25] stated the cell types (Balb/c3T3, ROS 17/2.8, L 929, WI-38) react differently to dental materials. It was concluded that the cytotoxicity of cast alloys and released ions is significantly affected by which cell line is selected for the test. The cell type used in the present study is significantly important for the tests.

Schedle and et al[16] indicated metal ions at different concentrations had an important cytopathogenical effect on L 929 fibroblasts, human gingival fibroblasts and mast cells at every concentration level.

Hornez and et al[30] found that Au, In, Sn, Pt, Ti, Pd ions had the lowest toxicity even at high concentrations. Cr, Ag, Cu were described as toxic and Ni, Co, Zn ions had a high level of toxicity.

In our study, at the diluted and concentrated solutions, Be, Ag, Cu had the highest toxicity followed by Ni, Cr, Co, Zn after 72 hours. The lowest toxicity level was observed in Mo.

In our previous study, the citotoxicity of dental casting alloys was assessed regarding the morphology of actin microflaments, intermediate filaments (vimentin) and microtubules. The detrimental effects on Ni-Cr alloys were higher than those of Co-Cr alloys. It would seem reasonable to conclude that in Ni-Cr alloys the severity of actin-based cellular functions depends on the cyototoxic potential of nickel ions released by corrosion.[31]

As a result, when metal ion is incontact with cell, it has an deleterious effect even at low concentration. More sensitive cell culture tests and/or longer in vitro test periods are needed for evaluation of the responses of cultured cells to alloys and types of metal ions which are released from these alloys.

CONCLUSION

The elements and the concentrations responsible for in vitro cytotoxic effects is important because it will help in vitro tests and designing new alloys to avoid adding the elements that appear to be harmful.

Within the limitations of this study, metal ions effect on viability of L 929 mouse fibroblast. Each ion has a spesific effect mechanism. Concentrations of metal ion are important factor on cell viability. Concentrated range accelerates the cell necrosis.

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