# THE EFFECT OF NICKEL AS PRODUCT OF RECASTING NICKEL CHROMIUM ON GINGIVAL FIBROBLASTS APOPTOSIS

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**Abstract**

NiCr restoration recasting causes an increase of Ni solubility in artificial saliva. Nickel [II] can freely enter the cell and behaves as a free radical causing oxidative stress and inducing apoptosis.

The purpose of this study was to determine the highest [Ni] in the artificial saliva and its effect on apoptosis of gingival fibroblasts.

Three types of NiCr restoration are prepared (casting ; one-time recasting and two-times recasting). Each restoration consisted of 8 speciments, and was subsequently immersed in artificial saliva for 7 days. The highest [Ni] in each restoration was exposed to the gingival fibroblasts culture with a density of $10^5$, and incubated for 1, 3, and 7 days. Analysis of [Ni] was performed by AAS, whereas apoptosis was determined by the used TUNEL assay method.

The highest [Ni] on restoration casting was 0.780 µg/L; while one-time recasting was 3.002 µg/L, and two-times recasting was 6,320 µg/L. There was an interaction between increased Ni solubility and exposure duration to apoptosis of gingival fibroblasts ($p< 0.0001$). The greatest mean ± SD of apoptosis was on the exposure of 6,320 µg/L with an incubation period of seven days ( 62.13 ± 2.85 ).

It can be concluded that the higher [Ni] is exposed to gingival fibroblasts in cell culture, the higher the incidence of apoptosis. Nickel [II] suspectedly enters into the cell via DMT – 1, which subsequently triggers apoptosis characterized by ROS generation and reduction of Bcl-2 expression.

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## Introduction

Nickel chromium (NiCr) alloys have been widely used in dentistry for denture construction, such as crown and bridge, and metal frame denture. The considerations for using it are due to adequate force, and good physical and mechanical properties. NiCr alloy is mainly composed of 68-80% Ni and 11.9 to 26.3% Cr, as well as other elements such as Nb, Mo, Be, Si, Al and Ti with variation from 0.1 to 14% [1].

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This alloy is manipulated by casting technique, although such failures often occur as distorsion, porosity and shrinkage. Improvements carried out with new alloy are costly and burdening to some patients. Due to the economic considerations, it can be considered for recasting[2].

Recasting causes property changes in alloy. The chemical changes that often occur is an increase in oxidation and corrosion level. The impact of corrosion in recasting NiCr restoration will increase the solubility of Ni and Cr cathions in saliva and cytotoxicity in gingival fibroblasts [3,4].

NiCr restoration releases corrosion products (NiO, $Cr_2O_3$) which may affect the morphology, viability and decreases cell proliferation. Ni exposure in cultured cells can decrease the activity of Fe-S enzyme that plays a

significant role in energy metabolism; induce oxidative stress through the formation of Reactive Oxygen Species (ROS), increase DNA damage response genes and induces apoptosis [5-7].

Apoptosis is an efficient mechanism to eliminate cells that are indesirable and may be harmful, so it can save the organism. Apoptosis is an active process, namely cell death via enzymatic digestion by it self. Apoptosis is regulated by existing of P 53 through activation of Bax, furthermore Bax inhibits expression of Bcl-2 [8,9].

This study will examine the effect of Ni as a corrosion product of recasting NiCr on gingival fibroblast apoptosis.

## Material and Methods

The design of this study was an experimental time series (Figure 1).

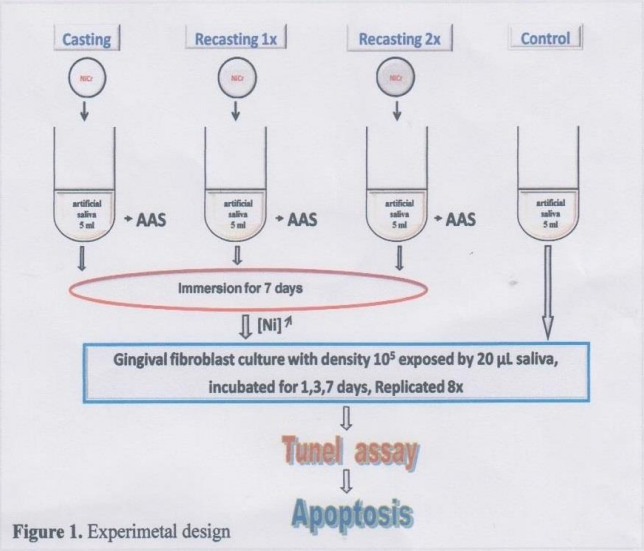

Figure 1. Experimetal design

It prepared three types of cylindrical NiCr restoration (diameter 10 mm, thickness 1 mm) obtained from casting NiCr alloy (NiCr 4all white ceramic alloy, Ivoclar Vivadent-USA). Each restoration had 24 speciments which were randomly-divided into 3 groups on each restoration. Each group on each type of restoration was immersed in 5 ml of artificial saliva (pH = 6,7) for 7 days. Artificial saliva containing the highest [Ni] on each restoration was exposed to gingival fibroblasts culture.

The control group was exposed only with artificial saliva in gingival fibroblasts. After conducting the procedure and filling in the informed consents, gingival tissue was obtained from the patients, and then gingivectomy was performed. The following stage was the processes of tissue culture. Cell cultures were grown in Dulbeco's Modified Eagle's Medium (DMEM) with Fetal Bovine Serum (FBS) containing 10% (v/v), 2 µM gluthamine, 100 U penicillin-streptomycin 1% (v/v).

Cells were harvested from the culture dish with trypsin-EDTA 0.025% (Gibco). Cells with a density of $10^5$ were grown on a coverslip in 96 wells. Furthermore, they were divided into 4 groups (@ 24 wells).

Each group was subsequently divided into 3 sub-groups (@ 8 wells). Each well was exposed to 20 µL of saliva containing the highest [Ni] in each restoration (incubated for 1, 3, and 7 days). Coverslip containing the cells was removed and placed on a glass object, then staining was performed.

Ni solubility was analyzed with Atomic Absorption Spectrophotometer (AAS) (Model AG analytikjena ZEEnit 700).

While apoptosis by terminal deoxynucleotidyl transferase-mediated method dUTP Nick End Labeling (TUNEL) was analyzed using the TUNEL Apoptosis Detection Kit for adherent cells (Biotin-labeled POD), Cat. No. L00296, GenScript USA.

A 400x light microscope (Olympus BX-50 Japan) magnification was used to observe cell apoptosis, which in the nucleus was conspicuous brown-blackish.

Percentage (%) of Apoptosis = number of apoptotic positive cells / total number of cells x 100%.

Analysis of the data applied the two-ways ANOVA, followed by Fisher's LSD multiple comparisons test with 95% level of significance ($\alpha = 0.05$).

This study has been approved by the ethical research committee of the Faculty of Medicine, Jember University, Number: 125/H.25.I.II/KE/2011.

## Results

The result of AAS conducted using a wavelength of 323 nm, the parameters per parts million (ppm) and the units used was µg/L. The highest of [Ni] on various restorations after immersion in artificial saliva for 7 days was [Ni] casting = 0.780; while for one-time recasting was

3.002 and two-times recasting procedure was 6.320 as shown in Table 1.

| Restorations | [Ni] (µg/L) |
|---|---|
| Casting | 0,780 |
| Recasting 1x | 3,002 |
| Recasting 2x | 6,320 |

**Table 1.** The highest of [Ni] on various restorations after immersion in artificial saliva for 7 days

Table 2 shows the results of two ways anova of apoptosis on the various [Ni] and duration of exposure, the means show a significant difference (p <0.0001), as well as the interaction between [Ni] and exposure duration (p <0.0001), and the coefficient of determination $R^2$ = 0.976, which means the ability of the independent variable ([Ni] and exposure duration) explains that the variant in the dependent variable (gingival fibroblast apoptosis) is approximately 98%. Figure 2 shows the immunocytochemistry determined under Tunel assay.

| Sources | df | Mean Square | F | p valued |
|---|---|---|---|---|
| Corrected model | 11 | 2568,397 | 359,650 | <0,0001 |
| Intercept | 1 | 78718,760 | 11022,923 | <0,0001 |
| [Ni] | 3 | 8260,483 | 1156,709 | <0,0001 |
| Days | 2 | 1499,448 | 209,966 | <0,0001 |
| Interaction [Ni]*Days | 6 | 78,670 | 11,016 | <0,0001 |

***Notes*** : *$R^2$ = .979 (Adjusted $R^2$ = .976)*

**Table 2.** Two ways anova of apoptosis on the various [Ni] and duration of exposure

Apoptosis gingival fibroblasts among [Ni] on the exposure duration presents significance difference (p<0.0001). The greatest mean ± SD (62.13 ± 2.85) of apoptosis is found in the exposure of 6,320 µg/L with exposure duration of 7 days as shown in Table 3.

| [Ni] (µg/l) | Duration of exposure (days) | | | p valued |
|---|---|---|---|---|
| | 1 | 3 | 7 | |
| 0 (control) | 5,63±0,52$^{a}$ | 8,50±1,60$^{b}$ | 11,38±2,9$^{c}$ | <0,0001 |
| 0,780 | 13,88±2,42$^{d}$ | 20,50±2,56$^{e}$ | 26,63±3,34$^{f}$ | <0,0001 |
| 3,002 | 26,63±2,26$^{g}$ | 33,50±2,20$^{h}$ | 42,13±3,40$^{i}$ | <0,0001 |
| 6,320 | 41,38±4,07$^{j}$ | 51,38±2,72$^{k}$ | 62,13±2,85$^{l}$ | <0,0001 |
| p valued | <0,0001 | <0,0001 | <0,0001 | |

**Notes** : different superscripts show significant differences (p<0,05) based on multiple comparisons test

**Table 3.** Mean ± SD of apoptosis on various [Ni] and duration of exposure.

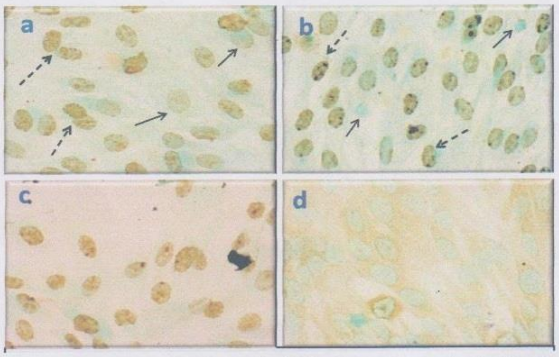


**Figure 2.** Apoptosis of gingival fibroblast (magnitude 400x)
Notes:
a. Exposure of 0,780 µg/L, incubated for 7 days, showed cells positive apoptosis ( - - → ) and normal cells ( ⟶ )
b. Exposure of 3,002 µg/L, incubated for 7 days, showed cells positive apoptosis ( - - - → ) and normal cells ( ⟶ )
c. Exposure of 0,780 µg/L, incubated for 7 days, showed all of the ve apoptosis and normal cells
d. Control group, incubated for 7 days, showed most of the cells are normal

## Discussion

NiCr alloy is used in dentistry for constructing dental restorations. The most common problem is the failure of the casting processes, and it can be corrected by recasting leading futher problem which is the occurrence of corrosion caused by the influence of oral environment. Corrosion is a deterioration phenomenon of materials due to their chemical reaction with intolerable environment. The mechanism of corrosion is an electrochemistry process that involves the transfers of electrons. NiCr restoration will take a reducing agent measure and $O_2$ dissolved in saliva acts as an oxidizer.

Passivity film of surface oxide on NiCr restoration can be deteriorated by corrosive environments. Anode on the surface of the restoration will release electrons, where the ions will be dissolved. Ni tends to be released into electrolyte in the form of $Ni^{++}$. Ni in the periodical table is the 28$^{th}$ element which is a transition metal. In the biosystem, Ni is generally found in the form of oxidized Ni ($Ni^{++}$) [10,11].

Fibroblasts, the cellular elements mostly found in the gingival connective tissue, are actively proliferating cells in normal circumstances, i.e. the regeneration processes or in the recovering and healing processes of tissue damaged. Ni exposure in fibroblasts is

responsible for oxidative stress and induces p53 in the nucleus [12,13].

Nickel is phagocyted by the cell on membrane ruffling region when there is contact between the Ni particles and surface membrane of the cell. Time required to start contact up to the endocytosis process of Ni is 7-10 minutes. The process is salvatory cell motion. Ni accumulation in and around the nucleus takes 24-48 hours [14].

The process of apoptosis is regulated via two pathways. The extrinsic (cytoplasmic) through the Fas death receptor activity by activating the Fas-Fas ligand interactions (FasL). The intrinsic pathway (mitochondria) that stimulate the release of cytochrome-c (cyt-c). This pathway depends on the regulation of Bcl-2 as an anti-apoptotic protein, and Bax as a pro-apoptotic protein. Apoptosis which is activated via the intrinsic pathway can be induced by oxidative stress and redox changes that in turn will assign a signal to the mitochondria to release cyt-c. Finally, cyt-c will activate caspase 9 which induce apoptosis [15,16].

In this study, the interaction between [Ni] and exposure duration on the apoptosis of gingival fibroblasts is found. The higher [Ni] and the longer the duration exposure are, the higher the apoptosis is. It is due to $Ni^{++}$ enters into the cell by means of competing with the essential trace elements ($Cu^{++}$, $Zn^{++}$, $Mn^{++}$, $Co^{++}$ and $Fe^{++}$), which is required for metabolic enzymes activity. In addition, $Ni^{++}$ has a high electrode potential allowing it to possess a high affinity for negative charge. Based on the foregoing, $Ni^{++}$ suspectedly enters into the cell through ion channel namely Divalent Metal Transporter-1 (DMT-1).

The corrosion progress of NiCr restoration in the physiological saliva is higher compared to those in artificial saliva. This is due to oral environment is more dynamic, such as viscosity, secretion volume, changes in pH, enzyme activity and low surface tension. In vivo, a number of biological factors including organic acids and enzymes produced by oral microorganisms contribute to the corrosion of restoration. Besides, the interaction between the restoration and specific factors in each individual can significantly affect corrosion. In addition, the presence of *Streptococcus sanguis* will lead to increased restoration corrosion inside the mouth [17,18].

Normally, in the cell there are secondary active transport ions, i.e. $Na^{+}/Ca^{++}$ exchanger that transfers three $Na^{+}$ for each single $Ca^{++}$ out of the cell. If the concentration of extracellular $Na^{+}$ is abundant. It will be compensated by mobilizing of intracellular $Ca^{++}$ into the extracellular, it causes the down-regulation of [Ca++] intracellular. Consequently, the compensation of intracellular $Ca^{++}$ occurs, especially that come from mitochondria, followed by the release of cyt-c, then triggers the occurrence of apoptosis [19].

Nickel [II] in the cells can increase the $OH_{*}^{-}$ generation through the Fenton and Haber-Weiss reactions. The higher [Ni] is, the more the formation of OH*- is accumulated in the cells possibly leading to oxidative stress, which antioxidant enzymes cannot eliminate. Furthermore, it provides a signal to the mitochondria which was initiated with the opening of the outer mitochondrial membrane, followed by matrix swelling and loss of mitochondrial membrane potentially leading to the release of cyt-c. Moreover, $Ni^{++}$ can be responsible for the DNA damage directly by making an attack on hydrogen bridges found between in the pair of purine and pyrimidine nitrogen bases [20].

Mitochondrial Permeability Transition Pore (MPTP) and apoptotic protein Bax are two important olygomers allowing the release of cyt-c. MPTP opening affects the accumulation of Ca, oxidants and down-regulation of potential mitochondrial transmembrane. MPTP is actually too small to be passed by cyt-c (13 KD), but merging with Bax, it forms a channel specifically for cyt-c. The merging of Bax with MPTP in the activity of pore formation is inhibited by anti apoptotic protein Bcl-2. Thus, the balance between pro-apoptotic proteins, anti-apoptosis and its interaction with MPTP determine the viability of the cells [21,22].

The release of cyt-c induces a series of events resulting in the activation of intracellular protease caspases group. Increased cyt-c in the cytoplasm forms apoptosom consisting of Apaf-1, cyt-c, dATP and aspartyl directed protease caspase 9. Interaction in apoptosom activates caspase 3. It binds to DNAse, then DNAse migrates towards the nucleus and initiate DNA damage [23,24].

The existing of extracellular Ni effects intracellular Ca transport through a calcium ionophore ionomicyn channel, thus Ni acts as a calcium channel blocker. The decrease in intracellular [Ca] will lead to an increase in free

Ca derived from the inventory in the cell. Changes in the intracellular concentration cause signaling changes in expression of apoptotic gene. Therefore, mitochondria serves as a buffer against Ca ions. Thus it can be presumed that the phenomenon of apoptosis caused by the discharge of the endoplasmic reticulum Ca is in conjunction with the release of cyt-c from mitochondria. Ca is identified as a messenger for the occurrence of apoptosis through caspases activity, cyt-c and nuclease enzymes [25,26].

Cyt-c plays a very essential role, i.e. as one of the important proteins in the electron transport system of the respiratory chain or terminal oxidation. In that processes, final oxidation occurs to produce energy in the form of ATP. Therefore, cyt-c release from mitochondria will affect the production of ATP level and trigger apoptosis [27].

Recasting of NiCr will increase corrosion, i.e. a release of $Ni^{++}$ in the artificial saliva. Nickel [II] diffuses into the gingival fibroblasts through specific ion channels, and stimulates the generation of ROS that causes DNA damage. DNA damage activates P53 in the nucleus. P53 stimulates and activates the DNA repair gene. If the process of DNA repair is not successful, then the P53 located in the nucleus is out into the cytosol. In the cytosolic, P53 will suppress Bcl-2 through activation of Bax, and eventually the cell will execute themselves through apoptosis mechanism.

## Conclusions

This study presents the effect of nickel as a corrosion product of recasting NiCr on gingical fibroblasts apaptosis in artificial saliva. The highest [Ni] occurs in the two-times recasting with seven days of immersion i.e. 6,320 µg/L. The higher [Ni] is exposed to the gingival fibroblasts and the longer the incubation is, the more cells undergo apoptosis. The greatest mean ± SD of apoptosis occurs in the exposure of 6,320 µg/L with incubation period of seven days (62.13 ± 2.85). Nickel [II] suspectedly enters the cell via DMT-1, and subsequently triggers apoptosis characterized by ROS generation, reduction of Bcl-2 expression and induction of Bax, cyt-c, and caspases (9 and 3).

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