

## Research Report

## The effect of nickel as a nickel chromium restoration corrosion product on gingival fibroblast through analysis of Bcl-2

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### ABSTRACT

**Background:** Restoration of NiCr may undergo corrosion process in artificial saliva. Corrosion product is soluble Ni substances in salivary electrolytes.  $Ni^{2+}$  may freely enter the cells through passive transport DMT-1.  $Ni^{2+}$  in the cell causes initiation of the ROS formation, which subsequently can conduct the redox reactions leading to DNA damage. The damage DNA affects the genetic expression, especially bcl-2, and even triggers apoptosis. **Purpose:** The aim of this study was to reveal the mechanism of Ni toxicity as a corrosion product of NiCr restoration on gingival fibroblasts through expression analysis of Bcl-2. **Methods:** Cells with a density of  $10^5$  planted on each coverslip in 72 wells to the treatment group and 24 wells to the control group (24 hours incubation). In the treatment groups, each well exposed with 20  $\mu$ L artificial saliva containing Ni concentration results immerse each restoration, whereas the control group was exposed to 20  $\mu$ L artificial saliva (incubation 1, 3, and 7 days). The data collected were subsequently analyzed using two-ways ANOVA, followed by one-way ANOVA. Comparing between experimental groups after one-way ANOVA was conducted using Fisher's LSD. Whereas, the calculation and documentation of Bcl-2 expression was performed camera of Olympus Microscope BX-50 Japan. **Results:** Statistical analysis of two-ways ANOVA showed the presence of interaction between the increasing Ni concentration and exposure duration on the expression of Bcl-2 gingival fibroblasts ( $p=0.021 < \alpha=0.05$ ). **Conclusion:** It can be concluded that the higher concentration of Ni exposed to gingival fibroblasts, and the longer incubation time will decreased Bcl-2 expression.

**Key words:**  $Ni^{2+}$ , DMT-1, DNA damage, Bcl-2

### ABSTRAK

**Latar belakang:** Restorasi NiCr dapat mengalami proses korosi di dalam saliva artificial. Produk korosi yang dihasilkan adalah substansi Ni yang terlarut di dalam elektrolit saliva.  $Ni^{2+}$  bebas dapat memasuki sel (fibroblas gingiva) melalui transport pasif DMT-1.  $Ni^{2+}$  di dalam sel menginisiasi pembentukan ROS, yang selanjutnya dapat menjalankan reaksi redoks dan dapat menimbulkan kerusakan DNA. DNA yang rusak mempengaruhi ekspresi genetik, terutama Bcl-2 dan bahkan dapat memicu apoptosis. **Tujuan:** Tujuan penelitian ini adalah untuk mengungkap mekanisme toksisitas Ni sebagai suatu produk korosi restorasi NiCr pada fibroblas gingiva melalui analisis ekspresi Bcl-2. **Metode:** Sel dengan kepadatan  $10^5$  ditanam pada tiap-tiap coverslip di dalam 72 well untuk kelompok perlakuan dan ditanam pada tiap-tiap coverslip di dalam 24 well untuk kelompok kontrol (inkubasi selama 24 jam). Pada kelompok perlakuan, masing-masing well dipapar dengan 20  $\mu$ L saliva artificial yang mengandung konsentrasi Ni hasil perendaman tiap-tiap restorasi, sedangkan pada kelompok kontrol dipapar 20  $\mu$ L saliva artificial (inkubasi 1,3 dan 7 hari). Data yang terkumpul selanjutnya dianalisis menggunakan ANOVA dua arah dan ANOVA satu arah. Perbandingan antar kelompok eksperimental setelah analisis ANOVA satu arah menggunakan uji Fisher's LSD. Penghitungan jumlah sel yang mengekspresikan Bcl-2, kemudian dilanjutkan dengan dokumentasi dengan menggunakan kamera Olympus Microscope BX-50 Japan. **Hasil:** Analisis statistik ANOVA dua arah menunjukkan adanya interaksi antara peningkatan konsentrasi Ni dan lama paparan terhadap ekspresi Bcl-2 fibroblas gingiva

( $p = 0,021 < \alpha = 0,05$ ). **Kesimpulan:** Dapat disimpulkan bahwa semakin tinggi konsentrasi paparan Ni pada fibroblas gingiva dan semakin lama masa inkubasi, maka akan menurunkan ekspresi Bcl-2.

**Kata kunci:** Ni<sup>2+</sup>, DMT-1, DNA damage, Bcl-2

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## INTRODUCTION

Nickel-chromium (NiCr) alloy was widely used in dentistry for restoration construction purposes requiring strength, e.g. crowns, bridges and metal frame dentures. The consideration of the use of these alloys is mainly due to their relative low price compared to noble alloy, possess adequate hardness, and fine physical and mechanical properties. NiCr alloy consists of main components of Ni 65%, Cr 22.5%, as well as additional components of Mo, Fe, Si, Ce and Nb.<sup>1-3</sup>

Biocompatibility NiCr restoration is a critical issue because of its existence in the long term of intimate contact with oral tissue. The presence of water, ions in saliva, changes in temperature and pH are factors that play a role in the occurrence of tarnish and corrosion. This was correlated with the release of alloy elements that impacts on unfavorable the biological effects.<sup>4</sup> Corrosion may cause chemical changes of NiCr restoration. Chemical change occurring was an increase in restoration oxidation i.e. the formation of metal oxides easily detached from the surface of the restoration becoming cations. NiCr restoration recasting results in the increased toxicity in gingival fibroblasts due to the increase of Ni cations release in saliva.<sup>5,6</sup>

Ni is an unstable transition class and easy-released into saliva.<sup>7</sup> NiCr alloy potentially causes toxic effects, for the reason that it may cause cellular damage. Ni elements released may affect the ability of cellular metabolism, particularly proliferation.<sup>8</sup> Ni exposure on cultured cells may decrease the activity of several Fe-S enzymes associated with energy metabolism and induces oxidative stress by increasing the Reactive Oxygen Species (ROS). Generation of ROS in the cells affects the expression of B-cell lymphoma-2 (Bcl-2).<sup>9-11</sup>

Bcl-2 was a negative regulator of cell death, extends cell apoptosis and inhibits cell cycle. Bcl-2 family and the products were identified as a key regulator in the process of apoptosis in many cell types. Excessive expression of Bcl-2 increases cell survival. Apoptosis induced by Ni<sup>2+</sup> is marked by the decrease of the expression of anti-apoptotic protein Bcl-2 and Bcl-xL, and induces the increase of the expression of Bad, Bcl-xS, Bax, and caspase sit c (9,3 and 6).<sup>12</sup> Ni<sup>2+</sup> accumulation in cells can increase DNA damage response genes, decreased the expression of Bcl-2 gene and triggers apoptosis.<sup>13</sup> Therefore, this study aims to determine the effect of toxicity of Ni as a product of NiCr restoration corrosion results in gingival fibroblasts through analysis of Bcl-2 expression.

## MATERIALS AND METHODS

This study began with a laboratory test to obtain a sample i.e the concentrations of Ni were dissolved in 5 ml of artificial saliva after immersion of restoration NiCr casting, recasting 1× and 2× for 7 days.<sup>14</sup> Restoration NiCr (diameter of 10 mm, thickness of 1 mm) obtained from casting alloys NiCr (4all NiCr alloy white ceramic, Ivoclar Vivadent-USA). Ni concentrations were obtained with the Atomic Absorption Spectrophotometer (AAS) assay (Model AG analytikjena ZEE nit 700), subsequently exposed on gingival fibroblast culture. Negative control group (describes artificial saliva containing no Ni on gingival fibroblasts).

Gingival obtained from patients after conducting some procedures and filling out informed-consent, and subsequently undergoing gingivectomy. This study was approved by the ethical appropriateness of the research ethics committee of the Faculty of Medicine, Jember University Number: 125/H.25.I.II/KE/2011.

The next stage was to conduct the process of tissue culture. Cell cultures were grown in Dulbecco, s Eagle.s modified medium (DMEM) fetal bovine serum containing (FBS) 10% (v/v), penicillin-streptomycin 1% (v/v). The cells were then harvested from the culture dish/flask with 0.025% trypsin-EDTA (Gibco). Cells with a density of 10<sup>5</sup> planted on coverslip in 72 wells as treatment group and 24 wells as control group (24-hour incubation), then the media were removed and washed with PBS. In the treatment group, each well was exposed to 20 μL artificial saliva containing Ni concentration resulted from the immersion of each restoration, whereas the control group was exposed to 20 μL artificial saliva (incubation 1, 3, and 7 days). Coverslip containing the cells were taken and placed on an object glass, then performed immunocytochemistry assay with Bcl-2 staining.

Expression of Bcl-2 gingival fibroblasts was performed by immunocytochemistry assay with monoclonal antibody anti Bcl-2 (Bcl-2 kit: Bioworld, Cat. No. BS1511, USA). In this study, observations were carried out using light microscope (Olympus BX-50 Japan) with magnification of 400 x, where the cells that expressed Bcl-2 in the cytoplasm was yellow-brown. Percentage of Bcl-2 = The number of positive cells Bcl-2 divided by the total number of cells x 100%.<sup>15</sup> The data were analyzed by two way ANOVA, followed by one way ANOVA and multiple comparison Least Different Significance test with 95% significance level ( $\alpha = 0.05$ ).

## RESULTS

Nickel solubility in artificial saliva performed by AAS with a wavelength of 323 nm, the parameters per parts million (ppm) and the units used are  $\mu\text{g/L}$ . The result is [Ni] casting = 0.780; recasting 1 x = 3.002 and recasting 2 x = 6.320 (Table 1).

The mean and standard deviation of Bcl-2 expression progressively decreased with increasing Ni concentration and increasing duration of exposure. Based on this interpretation, it was necessary to analyze using two way ANOVA toward the possibility of interaction between increasing Ni concentration and duration of exposure (Table 2 and Figure 1).

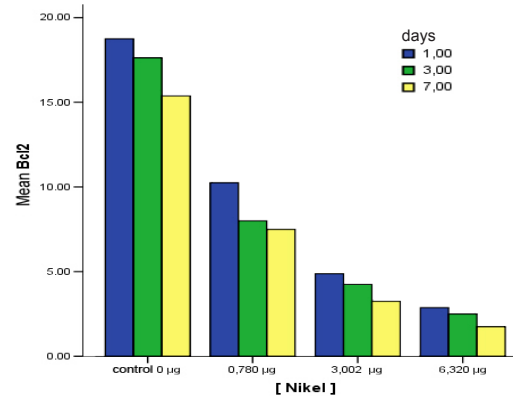
The result of two way ANOVA showed that there were significant differences in the mean of Bcl-2 expression among the different concentrations of Ni ( $p < 0.0001$ ), the mean expression of Bcl-2 also varied significantly among the durations of exposure ( $p < 0.0001$ ), as well as the interaction between Ni concentration and duration of exposure ( $p = 0.021$ ) (Table 3). The result of one way ANOVA showed that the expression of Bcl-2 gingival fibroblasts exposed to  $0\ \mu\text{g/L}$  (control), duration of exposure among the treatment groups are 1, 3 and 7 days, there was no difference between the mean of 1 day and 3 day-exposures, while there were significant differences in the mean of 1 day and 7 days, and the mean of 3 days and 7 days. Expression of Bcl-2 of gingival fibroblasts exposed to  $0.780\ \mu\text{g/L}$  shows that the mean of exposure duration of 3 days and 7 days was not significant difference, whereas the mean and standard deviation of exposure duration of 1 day and 3 days, as well as 1 day and 7 days demonstrate significant differences. In the mean time, expression of Bcl-2 gingival fibroblasts exposed to  $3.002\ \mu\text{g/L}$  shows that the mean exposure duration between 1 day and 3 days was not significant difference, whereas the mean of exposure duration between 1 day and 7 days, as well as 3 days and 7 days shows significant differences. Expression of Bcl-2

**Table 1.** [Ni] in the various restoration after immersion into artificial saliva for 7 days

Restoration	[Ni] ( $\mu\text{g/L}$ )
Casting	0.780
Recasting 1x	3.002
Recasting 2 x	6.320

**Table 2.** The mean and standard deviation of Bcl-2 expression in the various [Ni] and duration of exposure

[Ni] ( $\mu\text{g/l}$ )	$\bar{X} \pm \text{SD}$		
	Duration of exposure (days)		
	1	3	7
0	18.75 $\pm$ 1.49	17.63 $\pm$ 1.41	15.38 $\pm$ 1.69
0.780	10.25 $\pm$ 1.04	8.00 $\pm$ 1.07	7.50 $\pm$ 0.93
3.002	4.88 $\pm$ 1.13	4.25 $\pm$ 0.46	3.25 $\pm$ 0.71
6.320	2.88 $\pm$ 0.83	2.50 $\pm$ 0.76	1.75 $\pm$ 0.89



**Figure 1.** The mean and standard deviation of Bcl-2 expression in the various [Ni] and duration of exposure.

of gingival fibroblasts exposed to  $6.320\ \mu\text{g/L}$  shows that the mean of exposure duration between 1 day and 3 days was not varied, whereas the mean of exposure duration between 1 day and 7 days, as well as between 3 days and 7 days demonstrates significant differences (Table 4 and Figure 2).

## DISCUSSION

Rust was the result of corrosion, a form of metal oxides. Corrosion or rusting process was an electrochemical process. Restoration of NiCr acts as a reductant and  $\text{O}_2$  dissolved in the saliva acts as an oxidant. Rust formed on restoration accelerates the subsequent corrosion process, therefore, rust was also known as autocatalysis. Oral environment was very conducive to the formation of tarnish and corrosion, as always wet and pH changes. Corrosion occurs when elements in the restoration of the lost electrons and ionized, so that the ions are formed is released into the saliva. Corrosion reactions in the oral cavity was aqueous corrosion.

Genetic expression was an essential process activity in living organisms, as this process was the disclosure by the genes in the form of proteins, both structural proteins and protein enzymes. Structural support protein cellular components to perform its function, while protein enzymes catalyze change various compounds, including the genetic expression itself. Thus the process was the process of

**Table 3.** The result of two way ANOVA of Bcl-2 expression in the various [Ni] and duration of exposure

Source	df	Mean square	F	P valued
Corrected model	11	298.030	252.874	<0.0001
Intercept	1	6272.667	5322.263	<0.0001
[Ni]	3	1060.250	899.606	<0.0001
Days	2	39.385	33.418	<0.0001
Interaction [Ni]*Days	6	3.135	2.660	0.021

$r^2 = 0.967$

**Table 4.** The result of one way ANOVA of Bcl-2 expression in the various [Ni] and duration of exposure

[Ni] ( $\mu\text{g/l}$ )	Duration of exposure (days)			P valued
	1	3	7	
0	18.75 $\pm$ 1.49a	17.63 $\pm$ 1.41a	15.38 $\pm$ 1.69b	0.001
0.780	10.25 $\pm$ 1.04c	8.00 $\pm$ 1.07d	7.50 $\pm$ 0.93d	<0.0001
3.002	4.88 $\pm$ 1.13e	4.25 $\pm$ 0.46e	3.25 $\pm$ 0.71f	0.002
6.320	2.88 $\pm$ 0.83g	2.50 $\pm$ 0.76g	1.75 $\pm$ 0.89h	0.038
P valued	<0.0001	<0.0001	<0.0001	

**Note:** Vary of letter vary of significance (p valued<0.05) based on the result of multicomparison test

translating genetic expression of genes in the sequence of nitrogen bases form a protein amino acid sequence. Bcl2 gene is one of the genes that contribute to carcinogenesis. The role of the expression of Bcl-2 inhibits apoptosis by protecting mitochondrial transition pore, so that the sit-c was not released. The role of Bcl-2 expression was in contrast to P 53, if P 53 is active, the Bcl-2 was inactive, whereas if P 53 is inactive Bcl-2 was active, so that apoptosis does not occur, and proliferation continues. This study suggests that there was the interaction between the Ni concentrations with the exposure duration on the expression of Bcl-2.

The exposure to artificial saliva within 7 days may decrease the expression of Bcl2. It was caused by the anion present in the electrolyte, especially  $\text{Cl}^-$  may affect  $\text{Fe}^{2+}$  transport into cells by forming  $\text{FeCl}_2$  compounds. It may cause disruption in the activities of Fe (II)-dependent enzyme that was important in aerobic catabolism in the mitochondria that was aconitase converting citrate into

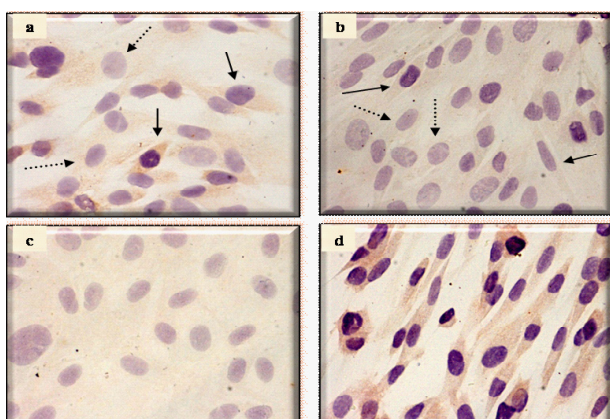
isocitrate leading to decreased ATP production. Disruption of mitochondrial function is followed by a decrease or loss of expression of Bcl-2.

Transport  $\text{Ni}^{++}$  into cells may disrupt  $\text{Fe}^{++}$  homeostasis by inhibiting the extracellular iron into the cell, thereby inhibiting iron-dependent enzymes in the cytoplasm and mitochondria.  $\text{Ni}^{++}$  may also replace co-factor of  $\text{Fe}^{2+}$  in the prolyl hydroxylases, an enzyme playing a role in the activation of HIF-1 $\alpha$ .<sup>16</sup> Ni increases the activity of malate dehydrogenase (MDH) and isocitrate dehydrogenase (IDH). Increasing MDH and IDH reflect that there is an attempt to restore the function of the citric acid cell cycle. It was also explained that Ni exposure may increase the activity of glycolysis and the ratio of NADH/NAD. Cell oxidative phosphorylation depends on the iron to transfer electrons from NADH to oxygen, oxidative phosphorylation disruption result in the accumulation of NADH. Disruption of iron homeostasis results in ATP depletion and decreased expression of Bcl-2.<sup>11</sup>

Bcl-2 plays an important role in the regulation of programmed cell death apoptosis that was genetically regulated, where it was a balance between the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax. It showed a high degree of homology to Bcl-2. Over-expression of Bax leads to heterodimer bond with Bcl-2, as a result, Bcl-2 functions become inhibited and apoptosis induced. Whereas, if Bcl-2 was over-expressed, the protein will be bond with each other in homodimer, causing the cell to survive.<sup>17</sup>

The process of apoptosis is regulated through two pathways, extrinsic pathway (cytoplasm) through the activity of Fas death receptor by activating the Fas-Fas ligand interactions (FasL), and intrinsic pathway (mitochondria), which triggers the release of sit-c which depends on the settings of Bcl-2 protein as an anti-apoptotic protein and Bax as pro-apoptosis protein.<sup>18</sup>

There are two key components that cause the release of sit-c, i.e. Mitochondrial permeability transition pore (MPTP) and apoptotic protein Bax. MPTP was located at the meeting point between the outer membrane and the inner mitochondrial membrane, which in an open state allowing compounds with a molecular weight less than 1.5 KD pass freely between the matrix and the cytosol. The opening of MPTP is affected by Ca accumulation, oxidant

**Figure 2.** Bcl-2 expression (magnitude 400x).

Notes:

- Exposure of 0.780  $\mu\text{g/L}$ , incubated for 7 days, showed cells strong positive expression ( $\longrightarrow$ ) and negative expression ( $\cdots\longrightarrow$ );
- Exposure of 3.002  $\mu\text{g/L}$ , incubated for 7 days, showed cells weak positive expression ( $\longrightarrow$ ) and negative expression ( $\cdots\longrightarrow$ );
- Exposure of 6.320  $\mu\text{g/L}$ , incubated for 7 days, showed all of the cell negative expression;
- Control, incubated for 7 days, showed all of the cell strong positive expression.



and low mitochondrial transmembrane potential. MPTP is actually too small to be bypassed by *sit-c* (13 Kd), but bonding with Bax forms a channel specifically for *sit-c*. The bonding between Bax and MPTP in pore formation activity is inhibited by anti-apoptotic protein Bcl-2. So the balance among pro-apoptotic protein, anti-apoptosis and their interaction with MPTP largely determine the cells viability.<sup>19</sup>

Phagocytosis Ni by cells in the region of membrane ruffling, when there was a contact between Ni particles and the surface of cell membranes. The time required from the first contact up to the Ni endocytosis into cells is varied, it takes about 7–10 minutes. That process shows the effort salvatory motion of cells. Ni accumulation in and around the cell nucleus takes about 24–48 hours. Ni inside the cell may cause DNA fragmentation directly or indirectly through the formation of ROS. Damaged DNA stimulates the P53, which will further suppress the expression of Bcl-2 via induction Bax.<sup>20</sup>

Ni compounds dissolved may affect intracellular Ca transport through a calcium ionophore ionomycin channel, thus Ni was known as calcium channel blockers. Decrease in intracellular Ca concentration would lead to an increase in the compensation process from the free Ca supplies inside the cell (endoplasmic reticulum and mitochondria). Changes in the intracellular concentration signals the changes in the expression of Bcl-2.<sup>21,22</sup> Ca possesses very important role, especially, its responsibility to regulate various physiological processes as well as involvement in maintaining homeostasis in pathological conditions and immune system. Therefore, it was also mentioned that the mitochondria serve as a buffer against Ca ions. Ca exits from endoplasmic reticulum along with *sit-c* release from mitochondria which was the phenomenon of apoptosis. Ca is identified as a message carrier to coordinate mitochondrial and endoplasmic reticulum that interact on apoptosis through suppression of Bcl-2, caspase activity, *sit-c* and nuclease enzyme.<sup>23,24</sup>

Statistical analysis shows the determination coefficient of expression Bcl2 ( $r^2 = 0.967$ ). Thus, in this case the ability of the independent variables (Ni concentration and duration of exposure) in explaining the variance of the dependent variable (Bcl-2 expression gingival fibroblasts) amounted to 97%, while the remaining 3% of variance of the dependent variable was caused by other factors.

Viewed from the size of the particles of each metal constructing NiCr alloys, then all of them may diffuse into the cell, because the size was smaller than the ion channels or pores of the membrane (300–400 Å), which has a negative charge. When viewed from the electrode potential, the ability of the metal to retain or release electrons, the  $Ni^{2+}$  enters first because it possesses the highest electrode potential. The electrode potential possessed by  $Ni^{2+}$ ,  $Ni^{2+}$  causes a higher affinity for electrons or negative charge than the other cations. Based on this, it may be presumed that the  $Ni^{2+}$  enters into cells via Ion channel divalent metal transporter-1 (DMT-1). The presence of  $Ni^{2+}$  in the cell may

replace co-factor in the cytosolic enzyme, especially  $Mg^{2+}$  co-factor required by G6PD. Eviction co-factor of  $Mg^{2+}$  by  $Ni^{2+}$  causes the enzyme catalytic function was lost, so the anaerobic catabolism in the cytosol may not undergo, so that the next process in the mitochondria for aerobic catabolism may not undergo either, and make the ATP as a source of cellular energy may not be generated.

The situation mentioned above may reduce the expression of Bcl-2. Another possibility was  $Ni^{2+}$  within cells act as free radicals that can cause oxidative stress in DNA. Injured/damaged DNA activates P 53 in the nucleus. P 53 stimulates and activates DNA repair genes. If the process of DNA repair does not succeed, then the P53 in the nucleus may be out into the cytosol. In the cytosol, P 53 will suppress Bcl-2 via activation of Bax, and eventually the cells will execute themselves through apoptosis mechanism.

$Ni^{++}$  diffuse into the gingival fibroblasts presumably through DMT-1. In the cells,  $Ni^{++}$  directly or indirectly, through stimulation of ROS formation, cause injury/damage DNA. The injured/damaged DNA leads to the suppression of Bcl-2 with Bax activation, and eventually the cell executes themselves through the mechanism of apoptosis. It can conclude that the higher concentration of Ni exposed to gingival fibroblast, and the longer incubation time will decreased Bcl-2 expression.

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