

## The Effect of NaF on the Existence of Ameloblasts and the Change of Tooth Dimension

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### Abstract

Ameloblasts cell which is very sensitive to the influence of internal and external factors. One external factor is the effect of fluoride elements that are able to affect the integrity of the ameloblasts cells and structure as well as the dimensions of the tooth enamel. This study seeks to analyze the effect of sodium fluoride exposure on the existence of tooth ameloblasts cells using indicators such as caspase-3, B-cell lymphoma 2 (Bcl-2), the distance between ameloblasts cells, as well as changes in the size or teeth dimension. Adult male healthy rats were used to experiment models, control (sterile distilled water treated), treatment group in to fluoride treated. The difference test on Bcl-2 and Caspase-3 between the control and the treatment groups showed different results ( $p < 0.05$ ). Similarly, the difference test on the distance between the ameloblasts cells as well as that on the dimensional change in both groups indicated significantly different results ( $p < 0.05$ ).

These results suggest that the induction of sodium fluoride showed the increase of Caspase-3 protein expression, the decrease of Bcl-2 expression, and the widening of the distance between ameloblasts cells, and the changes in the size or teeth dimension.

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### Introduction

Ameloblasts cells are cells derived from the ectodermal layer of epithelial tissue of the oral cavity. The role of ameloblasts cell on teeth growth takes place after predentin layer shaped by odontoblasts cells is formed. The growth process involving ameloblasts cells last for a certain period and the number of ameloblasts cells decrease after the tooth experiences enamel maturation and eventually degrade and disappear after tooth eruption.<sup>1,2</sup> In the process of growth ameloblasts cells undergo several stages and differentiation to achieve maturation, which plays a role in modulating and shaping the process of enamel matrix mineralization.

Fluoride is used to increase the hardness of enamel and to prevent dental caries through

substitution and incorporation into hydroxyapatite crystals in enamel. Fluoride is not produced by the body and is available in several forms of supplements.<sup>3</sup> Despite the advantages of fluoride, studies of the impact of fluoride on several organs especially ameloblasts cells are required. Impacts on the ameloblasts cells particularly in the secretory phase are related to a number of proteins associated with the growth of dental enamel, some of which are the main protein, i.e. amelogenin protein, as well as ameloblastsin, enamelin and secretory enamelsyn protease<sup>4</sup> a condition which may affect the maturation of hydroxyapatite crystals.<sup>5,6</sup> Another significant impact of fluoride exposure is the damage and/or death of ameloblasts cell. Certain amount of sodium fluoride in drinking water can cause apoptosis in ameloblasts cells from transitional period to enamel maturation.<sup>7</sup> Sodium fluoride can also induce cell death through the activity of certain proteins such as Bax and Bcl-2.<sup>8</sup> Besides such effects, fluoride also influences tooth morphogenesis. As stated in a study by Saglam et.al., the teeth dimensions in mesio-distal direction between normal children

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and those having dental fluorosis was not significantly different, except for some specific tooth such as lower jaw premolar.<sup>9</sup>

The current study uses a number of parameters: the expression of caspase-3 and Bcl-2 proteins, the change of teeth dimension, and the distance between ameloblasts cells. It aims at analyzing and demonstrating the impact of fluoride exposure on the existence of ameloblasts cells. This study was not conducted on humans for ethical reasons but it was conducted on experimental animals on male Wistar rats (*Rattus norvegicus*).

### Materials and methods

The study was experimental and the research design employed was randomized post-test only control group. The model used in this study was male *Rattus norvegicus*, aged 10-11 weeks, and weighed 150-170 g. The rodents were divided into two groups, each of which consisted of 10 rats taken randomly. The first group (control group) was given 2 ml of sterile distilled water via feeding tube, whereas the second group was given sodium fluoride/NaF (6.75 mgr in 2 ml of sterile distilled water). The amount of fluoride dose exposed was the optimal dose for the animal.<sup>10,11,12</sup> Analysis was carried out after 28 days of induction including some aspects, i.e. caspase- 9, Bcl-2 protein expression, by using monoclonal antibodies (anti-caspase-9 and anti-Bcl-2) measured by counting the number of ameloblasts cells that provided positive reaction. The expression of both proteins was done through immunohistochemistry coloring technique calculated per 10 High Power Field (HPF) and observed through light microscope with a magnification of 400 times.<sup>13,14</sup>

Another unit of analysis used was cell density measurement by gauging the distance between the ameloblasts cells (pm) in both control and treatment groups (Software: Cell-D). Digital Dental Calipers (mm) were used in the analysis of dimensional change of cervico-incisal and mesio-distal direction on mice mandibular incisors. Statistical analysis was used to analyse the difference of each variable between control and treatment groups using Independent T-Test, whereas Pearson correlation test was used to analyse several variables that affect the study.

This research was approved by *Animal Care and Use Committee*, a research ethic

committee in the Faculty of Veterinary, Universitas Airlangga, under ethical clearance No. 191- KE.

### Results

This study revealed that the exposure of sodium fluoride (NaF) to *Rattus norvegicus*. NaF affected the mandibular incisor in forms of enamel hypoplasia and a change in ameloblasts cell structure. The effect was due to the exposure to fluoride which caused delay in the process of amelogenin protein degradation in mineralization phase, yielding enamel structure which was charged by the protein. The effect of fluoride also caused amloblast cell damage and death through apoptosis process. Cell death through the mitochondrial pathway was regulated by the role of the main proteins, which are Bcl-2 and caspase-3. The results showed that there were differences in the expression of the two proteins, both in the control group or in the fluoride induction group. The most noticeable difference was in the group exposed to fluoride (see Table 1, and Figure 1, 2, 3). The ameloblasts cell damage and death impacted on the widening distance between ameloblasts cells, especially those exposed to fluoride. The result of immunohistochemistry analysis of Bcl-2 and caspase-3 protein expression as well as the distance between ameloblasts cells.

Expression	Mean ± SD	Mean± SD	Mean ± SD	Tooth Dimension	
				A	B
Group	Bcl-2	Caspase-3	Distance of cell		
Control	12,27 ± 0,7424 <sup>a</sup>	3,17 ± 0,4083 <sup>b</sup>	56,1895 ± 1,9656 <sup>a</sup>	2,5720± 0,3251 <sup>a</sup>	12,9080 ± 0,3105 <sup>a</sup>
NaF induction	4,31 ± 0,4998 <sup>b</sup>	13,22 ± 0,3645 <sup>b</sup>	112,9820± 0,8895 <sup>b</sup>	1,8020 ± 0,1488 <sup>b</sup>	8,5650 ± 0,4803 <sup>b</sup>

**Table 1.** The mean and standard deviations (SD) of Bcl-2 and caspase-3 protein expression, the distance between ameloblasts cells and the dimensional change of the rat mandibular incisor

The observation on the NaF induction group showed lower amount of Bcl-2 protein expression but bigger amount of caspase-3 protein expression than those in the control group. The distance between ameloblasts cells was greater in the induction group compared to that in the control group, while the rats teeth in the treatment group had smaller size than those in the control group.

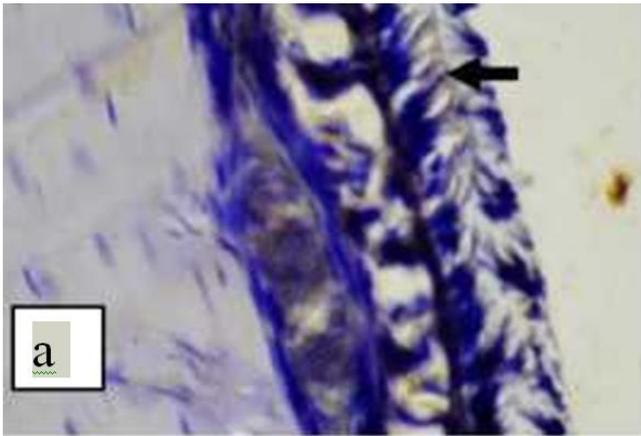


Figure 1a. Bcl-2 expression (Control group).

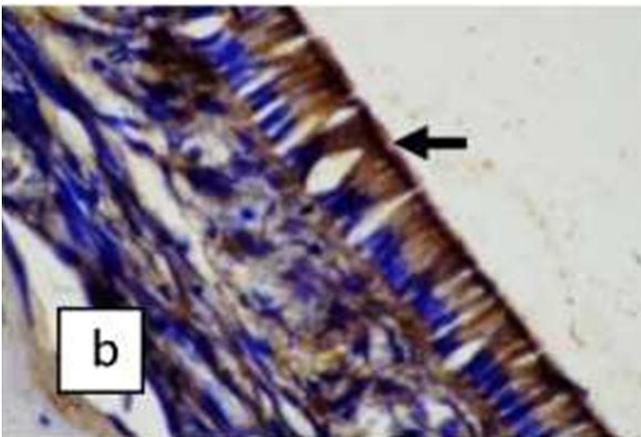


Figure 1b. Bcl-2 expression (NaF induction group)

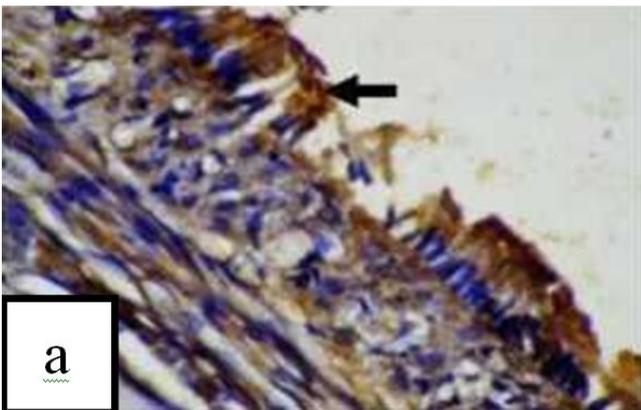


Figure 2a. Caspase-3 expression (control group).

The data normality test showed that all data were normally distributed ( $p > 0.05$ ). In the statistical analysis, the difference test on Bcl-2 and caspase-3 protein expressions between the control and the treatment groups showed different results ( $p < 0.05$ ). Similarly, the difference test on the distance between the

ameloblasts cells as well as that on the dimensional change in both groups indicated significantly different results ( $p < 0.05$ ).

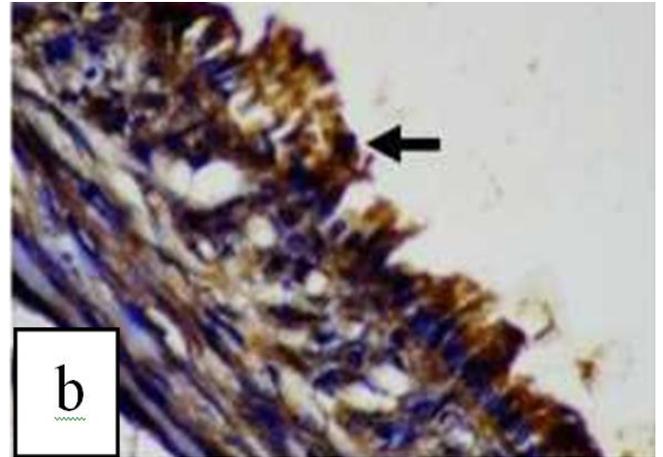


Figure 2b. Caspase-3 expression (NaF induction group).



Figure 3a. Distance between Ameloblasts cells (Control group).

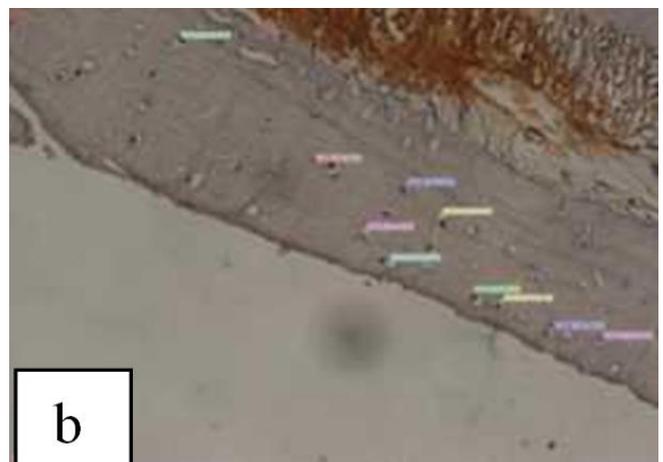


Figure 3b. Distance between Ameloblasts cells (NaF induction group).

The effect of fluoride exposure on the existence of tooth ameloblasts cells of Wistar rats using indicators such as caspase-3 protein and Bcl-2 protein expression, the distance between ameloblasts cells, as well as changes in the size or teeth dimension.

Some picture in above are an indicator from this research that has been done. It looks there a difference expression of protein such as Bcl-2 and Caspase-3 from the control group and the induction group of NaF. So then with expression of distance of ameloblasts cell.

The correlation test showed that there was no correlation between Bcl-2 and caspase-3 in the control group, but there was a significant correlation in the treatment group, with correlation coefficient value of 0.694. Similarly, the correlation test on the distance between ameloblasts cells with caspase-3 in the control group showed no correlation, whereas the test in the treatment group featured a significant correlation with correlation coefficient of 0.683. However, the test on the change in dimensions with caspase-3 showed no correlation, both in the control and treatment groups.

## Discussion

The result of this study adds to the previous research which stated that fluoride affects the growth of tooth enamel forming cell, namely ameloblasts, and confirms that fluoride also affects the dimensions and size of the teeth crown.<sup>15,16</sup> This research was conducted during the early phase of enamel matrix maturation, because it was expected to provide optimal manifestation. If the analysis were conducted on pre-maturation phase, the result would not be optimal since the structure of enamel matrix was not yet complete and could obscure the result of the analysis.

The research was also not conducted in the complete maturation phase since the density of enamel matrix would show an increased level in the result. As argued by Smith, the early stages of maturation requires 65% of total maturation period, a period when several proteins start to degrade. In the first growth phase of enamel maturation, the enamel matrix consists of 50% proteins and almost 49% minerals, whereas in the second growth phase the composition changes to 8% proteins and 65-70% minerals.<sup>17</sup>

The occurrence of dental fluorosis would affect the process of calcification and maturation of dental enamel so that teeth growth is not optimal. This has been confirmed in a number of studies stating that the main characteristics of fluoride-exposed teeth are hypo mineralization, large amount of amelogenin protein content, decreased levels of calcium, cell size appearing larger, and fragility of the ameloblasts cell subsurface. This condition also affects the teeth structure and anatomy, as found in Wahlujo's research in 2012 on 'the expression of Bcl-2, caspase-3, amelogenin, calbindin-28 kDa and enamel matrix density of ameloblasts cells as a result of sodium fluoride exposure with or without the addition of chloride calcium.<sup>18</sup> Exposure to fluoride also leads to cell death through apoptosis process marked in an increase in some proteins that regulate apoptosis, such as Bcl-2 and the executor in the apoptosis process, which is caspase-3. This study revealed that fluoride induction could cause apoptosis of ameloblasts cells, as proven from the NaF induction on the treatment group which showed that the number of Bcl-2 protein expression cells was lower than that in the control group and an increase in the number of caspase-3 protein expression cells as the regulator of apoptosis.

Bcl-2 protein is an anti-apoptotic protein located in mitochondria, endoplasmic reticulum and nucleus, while the pro-apoptotic proteins, i.e Bax, Bad, and Bid, are located in the cytosol. Apoptosis regulation, which is related to fluoride induction, is manifested in the form of Bcl-2 protein expression and also depends on the activity of p53 protein.<sup>19</sup>

P53 protein functions as an activator of transcription, which regulates the expressions of several pro-apoptotic proteins and is produced in response to intrinsic stress stimuli. Another function of the p53 protein is related to the regulation of cell division and DNA regulation of gene repair and gene apoptosis. If the gene repair fails, the p53 will serve as a mediator for gene activation of apoptosis regulator gene, which is Bcl-2 family (Bax) to open mitochondria pore in order to release cytochrome-c, which will further trigger cascade caspase.<sup>20</sup> Bcl-2 protein itself inhibits apoptosis by closing the mitochondrial pore allowing the cytochrome-c not to be released into cytosol.<sup>21</sup>

Ameloblasts cell apoptosis induced by fluoride is associated to mitochondrial response

to apoptosis. It is evident that the fluoride induction leading to ameloblasts cell death is through the mitochondrial pathway or intrinsic pathway. This can be explained as follows : (a) the induction of NaF causes the release of cytochrome-c from the mitochondria so that the amount of cytochrome-c will increase in the cytosol. This will further activate caspase-3 which acts as executor of apoptosis process, (b) induction of NaF would result in increased expression of the Voltage Dependent Anion Channel (VDAC) which is the main component of Mitochondrial Permeability Transition Pore (MPTP). Thus, there will be an increase of cytochrome-c in the cytosol, which will then activate caspase-3 protein. This result of study confirms the previous research conducted by Agalakova and Gusev.<sup>22</sup>

In this study the use of ameloblasts cells from rat tooth provides evidence that NaF induction also causes the increase in the number of caspase-3 protein expression cells. Two significant components in the release regulation of cytochrome-c from mitochondria to the cytosol are MPTP and pro-apoptotic protein Bax. MPTP is located at the meeting point between the inner membrane and the outer membrane of mitochondria which, in an open state, allow some compounds with a molecular weight of less than 1.5 kDa to pass freely from the matrix to the cytosol or the other way around.<sup>22</sup> Theoretically, it is unlikely for cytochrome-c with molecular weight of 13 kDa to pass MPTP, but the merger mechanism between MPTP and Bax will establish a certain channel for cytochrome-c to pass. Hence, Bcl-2 protein functions to inhibit the merger between MPTP with Bax.<sup>23</sup>

During the process of ameloblasts cell differentiation, two important physiological activities are endocytosis and phagocytosis. Fluoride exposure in relatively high doses would inhibit the function of endocytosis, influence and improve the function of phagocytosis.<sup>24</sup> Exposure to fluoride can also cause a decrease in the activity and reduction function of Matrix Metalloproteinase-20 (MMP-20), which is the mediator of enamel matrix remodelling. According to Sierant and Barlett, fluoride has no direct effect on the enzyme activity.<sup>3</sup> However, if there is an increase in fluoride levels, it will not directly reduce the activity of protease, but only inhibit the potential mechanism of protease. A decrease in the activity of reducing amelogenin

protein is primarily due to an increase of electro negative F ions which bind rapidly with three amino acid components contained in the amelogenin protein through hydrogen bonding. This will lead to changes in the alkalinity of the amino acid residues and will cause a decrease in intra-cell pH ranging from 5.5 to 5.6, while the activity of MMP-20 occurs at pH 7.2 to 7.3. This result consistent with the research finding by Den Besten et.al., and Uskokovic et al.<sup>25,26</sup> As a result of the delay of amelogenin protein degradation process, a great deal of the enamel will consist of protein and the mineralization process will be incomplete. Normal degradation process aims to provide free space on the enamel matrix which will then be used for enamel remineralization process. As a result, it will indirectly affect the structure and dimensions of the tooth enamel.

This study reveals that the mean distance between ameloblasts cells in the control group is smaller than that in the treatment group, as shown in Table 1. This is linked to the role of fluoride in increasing the occurrence of cell death through apoptosis by affecting mitochondrial function and pressing the number of cells that express Bcl-2 protein. The process will later inhibit apoptosis by closing the mitochondrial pore, allowing the cytochrome-c not to be released into the cytosol and increase the number of caspase-3 protein expression cells, the regulator of apoptosis as argued by Wahluoyo (2012) in a research entitled 'the expression of Bcl-2, caspase-3, amelogenin, calbindin-28 kDa and enamel matrix density of ameloblasts cells as a result of sodium fluoride exposure with or without the addition of chloride calcium'.<sup>18</sup>

Exposure to fluoride will alter cellular metabolism by inhibiting the secretion of several enzymes that function in the physiological processes of ameloblasts cells and some enzymes associated with cell production or energy cell, such as enolasephosphoglucomutase, succino dehydrogenase and cytochrome oxidase. In addition, there are two heme enzymes containing catalase and peroxidase that decrease H<sub>2</sub>O<sub>2</sub> produced in the cell. The state will interfere with cell hemostasis resulting in cell death and eventually will affect the number and distance of ameloblasts cells.<sup>27</sup>

Based on the results of this study, it is argued that fluoride exposure can cause ameloblasts cell apoptosis, widen the distance

between the cells, and change the dimensions of the rat teeth. The findings of this study may provide further understanding on the effects of fluoride exposure to ameloblasts cells. These results suggest that the induction of sodium fluoride showed the increase of Caspase-3 protein expression, the decrease of Bcl-2 expression, and the widening of the distance between ameloblasts cells, and the changes in the size or teeth dimension.

## Conclusions

Exposure to fluoride causes apoptosis of ameloblasts cells, the widening of the distance between the cells and the change of the rats teeth dimension.

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## Declaration of Interest

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