Evaluation of the Impact of Sodium Fluoride on Amelogenesis in Rattus Novergicus

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Abstract

Amelogenesis is driven by a number of exceedingly complicated elements, components, and processes. Each component, such as the Amelogenin protein and the proteinase MMP-20, performs critical and strategic functions. Fluoride exposure at particular doses and durations changes the amount of ameloblast cells necessary for the process of amelogenesis.

The results of this study showed that sodium fluoride induction accelerated the breakdown of fragments into smaller Amelogenin proteins, decreased the function of caspase-3 in the drawn-out process of apoptosis, and increased the involvement of MMP-20 and Calmodulin-28kDa in the process of protein degradation. The spacing between ameloblast cells and their density would stay the same because there was no substantial cell dying mechanism. However, the matrix of the tooth enamel exposed to fluoride would become denser.

The scientists found that causing sodium fluoride in fluoride-exposed teeth could maintain ameloblast cell viability and enhance dental amelogenesis.

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Introduction

Natural resources like plants and trace elements like fluoride are provided by the environment. Particularly during the amelogenesis period, fluoride is crucial for tooth growth and development. The complex and unusual process of amelogenesis consists of many interconnected parts. Amelogenin and other proteinases, the most significant of which is Amelogenin, play a crucial part in this regulation of amelogenesis.

Fluoride in excess has a negative effect on tooth growth and development, especially during amelogenesis. The most noticeable outcome that we typically see is enamel hypoplasia or fluorosis. Ameloblast cells' metabolism may be affected and disrupted by high fluoride levels, a finding from a prior study, and this could result in a prolonged apoptotic process that eventually kills the cells[1]. In addition to these modifications or anomalies, exposure to fluoride can result in major changes in the shape and size of the teeth as well as the quality of dental enamel. Several scientific studies have brought attention to this assertion[2].

Important cells, proteins, proteinases, as well as other elements, all play important roles in the creation and development of the enamel. Complex and acting in numerous steps or stages at once are the factors regulating the amelogenesis process.

Ameloblasts and enamel created during the insult period have long been recognized to undergo alterations when exposed to high doses of sodium fluoride supplied parenterally[3]. The micro radiographic examination of such changed enamel has shown that a double response

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occurs after each fluoride injection: a first-formed hypo mineralized layer and later a neighboring hypo mineralized zone. This coupled response occurs frequently. whether intraperitoneal or subcutaneous injections are administered[4]. According to Schour and Smith, formative ameloblasts are described as containing clumps of darkly stained globules or granules and huge vesicles. Fluoride administration during enamel maturation or following the completion of enamel production results in hypo mineralization[5].

Regarding the shape of the ameloblasts linked to this maturational disruption, very few information is provided. Fluoride-induced changes in the ultrastructure of developing enamel and ameloblasts have also been observed. Using a replication process using undecalcified enamel sections, Kruger noticed what appeared to be a delay in mineralization. He described how an amorphous-appearing zone with fewer and less-oriented apatite crystals temporarily disrupted the typical arrangement of enamel rods.

The middle third of the ameloblasts were reported as having abnormally big vacuolar structures, and the apical third as having many inflated mitochondria[6]. As soon as fluoride injections stop, Light microscopic sections of decalcified enamel show that normal enamel development has restarted[5,3]. The results of the cytologic abnormalities seen in the fluorotic ameloblasts or the fine structure of the altered enamel after the fluoride injections were stopped have not been described.

Ameloblast cells produce a number of secretory proteins, including the protein Amelogenin and one of the proteinases, matrix metalloproteinase-20, in addition to their function as early initiators (MMP-20). Previous research discovered a wide variety of proteinases involved in the procedure, most notably around 15 different MMPs; nonetheless, MMP-20 is the essential proteinase that directly contributes to the amelogenesis process. Due to the production of four proteins that are released into the enamel matrix, including Amelogenin (AMELX), which makes up about 90% of the organic matrix, Ameloblastin (AMBN), Enamelsyn (ENAM), and MMP-20, ameloblast cells are crucial for the development and expansion of enamel. Amelogenesis, the process through which tooth enamel develops and expands, is regulated by MMP-20 proteinase. A major factor in enamel production is MMP-20. It has been demonstrated that enamel produced without MMP-20 expression will be of lower quality and contain a loss of thickess around 30% and density loss around 50% was observed[7].

The aim of the study is to evaluate the potential and influence of sodium fluoride and control on Amelogenesis process along with its effect on ameloblast cells in fluoride exposed teeth. The expression of different proteins and proteinases, the comparison of the mineral composition in tooth enamel, the density of tooth enamel, and the spacing between ameloblast cells are only a few of the criteria and indicators that are used. The goal of the current study is to examine all developmental phases of enamel during and following many intraperitoneal injections of fluoride.

Materials and methods

Ethical statement

This experiment uses a randomised post-test control group design. Rattus novergicus male was the experimental animal model employed in this study due to ethical issues and the necessity of the data. 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.

Research design

Animal models for this study were 10–11 weeks old, weighing 150–170 grammes, and in good health. The 60 rats used in this investigation were divided into two groups. Just sterile aqua dest was given to the first group as a control. The second group received 2 ml of sterile distilled water with 6.75 mg of fluoride (MERCK, Kenilworth, NJ; product registration number 1.06449.0250).[8]. The United States National Research Council has approved the dosage given to experimental animals (2005).

The solution was then prepared using hot water in the same amount—2 ml—as the first two groups. The experimental animal models in each group were to be put to sleep and assessed in accordance with the objectives of the study after receiving therapy for 24 days. By monitoring the expression of numerous related proteins, including proteinase, amelogenin, caspase-3, and matrix metalloproteinase-20, the researchers were able to accomplish their objectives (MMP-

20). The expression of the two proteins and proteinases was measured, computed per 10 High Power Fields (HPF), and assessed using a 400x magnification light microscope using immunohistochemical labelling.

Results

Whole data after evaluation of normalcy was subjected for statistical analysis and comparison tables are presented as follows-

	Group	N	Mean	Std. Deviation	Std. Error Mean	Mean Difference	t	P Value
Calcium	Control	30	31.4743	3.07008	.56052	14.47645	19.201	.000
	NaF Induction	30	16.9979	2.76190	.50425			

Table 1. Comparison of Mineral composition of calcium was done between Control and NaF Induction.

When comparison of Mineral composition of calcium was done between Control and NaF Induction it was found that the difference in mean was 14.47 and this difference in mean was statistically significant ($p<0.05$) as shown in table 1.

	Group	N	Mean	Std. Deviation	Std. Error Mean	Mean Difference	t	P Value
Phosphate	Control	30	18.7682	2.52741	.46144	5.85936	10.521	.000
	NaF Induction	30	12.9089	1.70786	.31181			

Table 2. Comparison of Mineral composition of phosphate was done between Control and NaF Induction.

When comparison of Mineral composition of phosphate was done between Control and NaF Induction it was found that the difference in mean was 5.85936 and this difference in mean was statistically significant ($p<0.05$) as shown in table 2

	Group	N	Mean	Std. Deviation	Std. Error Mean	Mean Difference	t	P Value
Distance between Cells	Control	30	81.9931	5.22834	.95456	-22.46723	-7.040	.000
	NaF Induction	30	104.4603	16.68043	3.04542			

Table 3. Comparison of Distance between Control and NaF Induction.

When comparison of Distance between Control and NaF Induction was done it was found that the difference in mean was -22.46723 and this difference was statistically significant ($p<0.05$) as shown in table 3.

	Group	N	Mean	Std. Deviation	Std. Error Mean	Mean Difference	t	P Value
Porosities Percentage	Control	30	21.4583	3.34605	.61090	-21.46974	-18.143	.000
	NaF Induction	30	42.9281	5.55107	1.01348			

Table 4. Comparison of Porosity Percentage between Control and NaF Induction.

When Comparison of Porosity Percentage between Control and NaF Induction was done it was found that the difference in mean was -21.46974 and this difference was statistically significant ($p<0.05$) as shown in table 4.

Discussion

On the growth and development of dental enamel, fluoride has a significant effect. The delay in the protein amelogenin's degradation, which results in delays and interruptions in the mineralization of dental enamel, is one of the most obvious effects. One of the proteinases involved in the process of amelogenesis, MMP-20, will be disrupted by fluoride in addition to its effect on particular proteins. According to Agalocova and Gusev, exposure to fluoride negatively affects the ability of ameloblast cells to survive and, at some levels, causes cell apoptosis. To demonstrate the relationship between the density of ameloblast cells and the density of the enamel matrix, the spacing between ameloblast cells must be known. The mechanism of apoptosis depends on the protein caspase 3, which makes additional research in this study.

Amelogenin is an important protein that functions crucially during the amelogenesis phase before vanishing. Amelogenin is a protein that tooth enamel needs in order to harden. Proline, glutamine, and histidine residues are included in this protein, which has a molecular weight between 22 and 30 kDa. The length of the amelogenin protein does not change throughout the early phases of enamel growth and development, while there is still an enamel matrix. The following stage of development, however, causes it to quickly lengthen and fragment into smaller parts. The breakdown activity of amelogenin protein matrix fragments controls the biomineralization process, which initiates the mineralization of the enamel. To hasten the crystallization process, enzymatic crystals will fill the space left by the fragmented amelogenin protein. Minerals start to accumulate on the enamel at this point, which is the start of

mineralization. On how protein matrix fragments degrade, fluoride exposure has a substantial impact. The mineralization process is slowed down by fluoride, which also lowers the quality of the enamel by inhibiting proteinases, particularly MMP-20 [1,7]. Fluoride raises the electronegative F-ion level, which causes the amelogenin protein's amino acids to form hydrogen bonds with one another very quickly. The link will change the amino acid residue's fundamental makeup because fluoride has strong electronegative characteristics. MMP-20 activity is impacted at pH values of 7.2–7.3 by the transition from alkaline to acidic circumstances (pH 5.5–5.6).

Fluoride can decrease MMP-20's activity and ability to mediate enamel matrix remodeling. According to Sierant and Barlett, fluoride has no direct impact on the activity of enzymes; nevertheless, an increase in fluoride levels inhibits potential protease processes rather than directly decreasing the activity of existing proteases. Reduced activity to diminish amelogenin protein will easily bind to the three amino acid components of protein amelogenin via hydrogen bonds due to an increase in electronegative from ion F. According to Uskokovic, this circumstance will alter the amino acid residues' alkaline nature, resulting in a dip in intracellular pH between 5.5 and 5.6. The intracellular pH range between 7.2 and 7.3 is the optimal range for MMP-20 activity, and this pH value will restrict MMP-20 activity. Due to the delay in the process of amelogenin protein degradation, the enamel will be dense with protein, which will render the remineralization process ineffective. The degradation process tries to provide space in the enamel matrix for remineralization under normal circumstances. This will directly alter the size and shape of the tooth enamel as it grows.

The amelogenesis process of fluoride-exposed teeth was greatly impacted by the presence of the calbindin-28 kDa protein, which was typically detected at a percentage of about 33% in the cytoplasm of ameloblast cells. A protein called calbindin-28kDa that inhibits apoptosis and promotes calcium transport into various tissues, sensors, and buffering. Calcium influences the production and secretion of Amelogenin and regulates transcellular influx via mineral ions for crystal formation by deposition on the surface/basal layer in secretory ameloblasts via two different pathways: (a) influencing Amelogenin and transcellular influx via mineral ions for crystal formation by deposition on the surface/basal layer[9].

Calbindin-28kDa is essential for calcium transport because, in contrast to Calbindin-9kDa, which only has two arms and has numerous 1/50-1/100 or 0.02 percent EF-sites, it has a strong affinity for binding calcium via four sides/arms. This scenario is related to the process of creating crystals in the enamel matrix because mineralization happens during the early maturation of enamel, allowing access to the amalgamation of many crystals, culminating in the crystallization process and a more prominent and denser crystal structure. Following this, either Calbindin-12kDa, which only contributes to protein deposits, or amelogenin protein breakdown occurs, which is consistent with other research[10,11,12].

Both free calcium ions and calcium-bound complexes have a variety of functions in biological processes. The mineralization and remodeling of bones and teeth are two of the most important functions of calcium in its bound state. Although calcium (Ca+2) in ionized form and complex bonds can diffuse directly, renal function filters this state. Calmodulin and calcineurin are two examples of receptor proteins that intracellular calcium will rely on and bind to[13,14].

Absorption in the small intestine, resorption in the kidneys, and bone remodeling, or the exchange of calcium ions in the bones, are the three processes that control calcium homeostasis. In the small intestine, tight junctions allow for the paracellular and transcellular absorption of calcium. The kidney and small intestine are both involved in the metabolism of calcium. Similar to transcellular absorption in the intestine, transepithelial Ca+2 reabsorption occurs in the kidney and necessitates the presence of calcium buffering proteins such calbindin-28 kD and calbindin-9 kD.

Phosphate and several other minerals are other elements in addition to calcium, albeit they are only present in minor quantities. Using SEM-EDX and GC-MS, the chemical composition of the elements found in rat tooth enamel during the amelogenesis phase was evaluated. In this work, calcium, and phosphate—the primary minerals in tooth enamel and crucial components of the mineralization process during the amelogenesis

phase—were examined. Water and protein are examples of additional minerals that were discovered[15].

Additional proof of the presence of ameloblast cells can be obtained by measuring the space between cells. This is due to fluoride exposure, which will enhance sustained apoptotic activity, characterizing the level of cell death that occurs and cannot be reversed, and modify the spacing between ameloblast cells[2].

The immunohistochemistry method was used to examine the preparations and measure the spacing between ameloblast cells using a microscope and Cell-D software at a 400 times magnification. Figure 1 below shows the results of the investigation on cell distance.

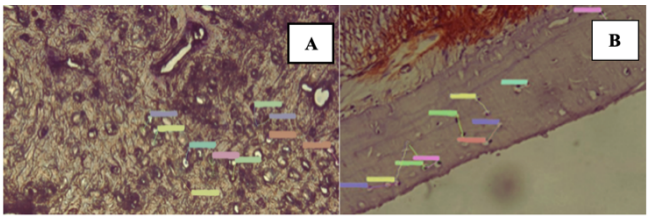

Figure 1. Distance between ameloblast cells in the control group (figure-A), induction of NaF (figure-B).

An overview of the separation between the two treatment groups of ameloblast cells is shown in the image above.

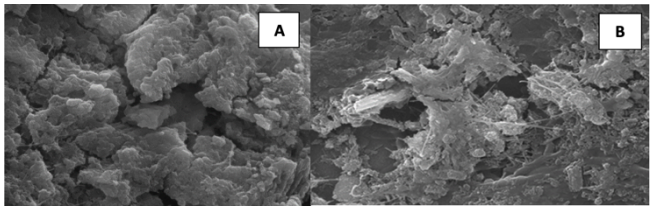

Figure 2. Rat tooth enamel density was examined using SEM in the control group (figure 2A) and afterNaF induction (figure 2B) at a 20.000x magnification.

Density is a marker of the amelogenesis process, which occurs as tooth enamel grows and develops. Researchers discovered a few years ago that fluoride exposure causes dental enamel to lose density. Figure 2B. This is because the enamel matrix is largely constituted of organic material, the bulk of which is the Amelogenin protein, which is not broken down by MMP-20 when exposed to fluoride. The numerous instances of porosity in the enamel matrix will be visible in SEM.

Porosity in the control group is a natural feature during the mineralization phase because organic material made up of water and some proteins makes up a minor fraction (4-10%) of the enamel matrix. In contrast to the inorganic materials, which appear as a bright or white colour, the organic material's black porosity in the enamel matrix creates a distinct image. The organic material of the enamel matrix provides a characteristic image on SEM analysis, notably the appearance of gaps or indentations between the matrixes with a black hue, which is compatible with Soares et al. In comparison, inorganic materials contain around 90% hydroxyapatite (Ca10 (PO4)6(OH) 2, giving the group without induction a bright or white look (Figure 2A). In SEM, a secondary electron signal is produced as a result of inelastic reflections, whereas a backscattered signal is produced as a result of elastic reflections[16].

Conclusion

This study concludes that sodium fluoride induction enhances growth and development, increases the presence of ameloblast cells, and improves the amelogenesis process in fluoride-exposed rat teeth. Several indicators can be concluded, including decreased caspase-3 expression and decreased Amelogenin protein expression, increased MMP-20 function in the process of Amelogenin degradation and increased calbindin-28 kDa, increased mineral composition of tooth enamel, decreased distance between ameloblast cells, and increased density of the enamel matrix tooth. In a nutshell, the present research establishes the heroic role of NaF in enamel formation in top to bottom processes of amelogenesis. Thereby, further studies are warranted to re-establish its role on human teeth.

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Declaration of Interest

The authors report no conflict of interest.

References

1. Wahluyo S, Ismiyatin K, Purwanto B, Mukono IS. The Influence of Sodium Fluoride on the Growth of Ameloblasts and Kidney Proximal Tubular Cells. Folia Biologica (Praha) 2017-A; 63:31 – 4.
2. Wahluyo S, Nuraini P, Rahayu RP, Ismiyatin K, Handayani R, Soetjipto. The Effect of NaF on the Existence of Ameloblasts and the Change of Tooth Dimension. Journal of International Dental and Medical Research 2017-B;10(1): 139.
3. Prasanth T, Saraswathi T. Histopathological and radiographic evaluation of rat molar teeth after traumatic injury-a pilot study. J Oral Maxillofac Pathol. 2012;16(3):313-7.
4. Aswini YB, Mohanty V, Rijhwani K. Fluoride and Other Trace Elements in Dental Hard Tissue. Human Tooth and Developmental Dental Defects - Compositional and Genetic Implications. IntechOpen; 2022. http://dx.doi.org/10.5772/intechopen.102043.
5. Castiblanco-Rubio, G. A. Martignon-Biermann, S. Castellanos-Parra, J. E, et al. Pathogenesis of dental fluorosis: biochemical and cellular mechanisms. Revista Facultad De Odontología Universidad De Antioquia 2017;28(2):408–421.
6. Kumar A, Choudhary MB. Morphological and ultrastructural effects of sodium fluoride toxicity on the liver of rat. J Environ Sci Eng. 2013;55(1):108-13.
7. Khan F, Liu H, Reyes A, et al. The proteolytic processing at Amelogenin, by enamel matrix-metalloproteinase-20 (MMP-20), is controlled by minerals ion; Biochim Biophys Acta 2015;(3): 2600 – 07.
8. Catani DB, Tenuta LMA, Andalo FA, et al. Fluorosis in rats exposed to oscillating chronic fluoride doses. Braz Dent J 2010;21(1):32–37.
9. Bolaños A, Hotton D, Ferbus D, et al. Regulation of calbindin-D(28k) expression by Msx2 in the dental epithelium. J Histochem Cytochem. 2012;60(8):603-10.
10. Bronckers ALJ, Lyaruu DM, DenBesten PK, et al. The impact of fluoride on ameloblasts and the mechanism of enamel fluorosis. J Dent Res 2009;88(10); 877–93.
11. Gopalakrisnan L, Doriya K, Santhos-Kumar D. Moringa oleifera: A review on nutritive importance and its medical application, Food Science on Human Wellness 2016;5 (2):49 – 56.
12. Nurbaeva MK, Eckstein M, Devotta A, Saint-Jeannet J-P, Yule DI, Hubbard MJ and Lacruz RS. Evidence That Calcium Entry into Calcium-Transporting Dental Enamel Cells Is Regulated by Cholecystokinin, Acetylcholine and ATP. Front. Physiol 2018. 9:801. doi: 10.3389/fphys.2018.00801
13. Munro P. Calcium Metabolism in Health and Disease. Clin J Am Soc Nephrol 2010;5: 523-30.
14. Tinawi M. Pathophysiology, Evaluation, and Management of Metabolic Alkalosis. Cureus. 2021;21;13(1): e12841.
15. Manggara AB, Shofi M. Analisis kandungan mineral daun kelor (Moringa oleifera Lamk) menggunakan Spektrometer XRF (X-Ray Fluorescence). Akta Komindo 2018;3(1):104 – 11.
16. Ozveren N and Ozalp S. Microhardness and SEM-EDX Analysis of Permanent Enamel Surface Adjacent to Fluoride-releasing Restorative Materials Under Cariogenic Challenges Oral Health Prev. Dent 2018; 16:417 – 24.