How pH is regulated during amelogenesis in dental fluorosis (Review)

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Abstract. Amelogenesis is a complicated process that concerns the interaction between growing hydroxyapatite crystals and extracellular proteins, which requires the tight regulation of pH. In dental fluorosis, the balance of pH regulation is broken, leading to abnormal mineralization. The current review focuses on the electrolyte transport processes associated with pH homeostasis, particularly regarding the changes in ion transporters that occur during amelogenesis, following exposure to excessive fluoride. Furthermore, the possible mechanism of fluorosis is discussed on the basis of acid hypothesis. There are two main methods by which F^- accelerates crystal formation in ameloblasts. Firstly, it induces the release of protons, lowering the pH of the cell microenvironment. The decreased pH stimulates the upregulation of ion transporters, which attenuates further declines in the pH. Secondly, F^- triggers an unknown signaling pathway, causing changes in the transcription of ion transporters and upregulating the expression of bicarbonate transporters. This results in the release of a large amount of bicarbonate from ameloblasts, which may neutralize the pH to form a microenvironment that favors crystal nucleation. The decreased pH stimulates the diffusion of F^- into the cytoplasm of amelobalsts along the concentration gradient formed by the release of protons. The retention of F^- causes a series of pathological changes, including oxidative and endoplasmic reticulum stress. If the buffering capacity of ameloblasts facing F^- toxicity holds, normal mineralization occurs; however, if F^- levels are high enough to overwhelm the buffering capacity of ameloblasts, abnormal mineralization occurs, leading to dental fluorosis.

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Contents

1. Introduction

Dental fluorosis is an enamel defect caused by the excessive intake of fluoride during enamel formation. In deciduous teeth, it occurs during the embryonic phase, while in permanent teeth, it occurs primarily in children aged 2-8 years old (1-4). The primary sources of fluoride intake are from food and water, as well as from toothpaste, which contains added fluoride. Fluoride is also being added to different materials, including fluoride varnish, fluoride foam and dental resin to prevent the occurrence of dental caries. All these methods increase the morbidity of dental fluorosis (1). The incidence of dental fluorosis is currently a problem worldwide, although the prevalence of dental fluorosis varies in different countries. In the USA, ~25% of the population have dental fluorosis and its incidence is also high in China, ~11.7% of adolescents of 12 years old suffer dental fluorosis (2,4). According to the Dean's index, dental fluorosis may be classified into five types: Questionable, very mild, mild, moderate and severe (5). In clinical practice, dental fluorosis may be classified into three types: Chalk, discoloration and defective (6).

Enamel formation by ameloblasts is a complex process. The primary elements of enamel hydroxyapatite are present in the crystalline form (7,8). It has been demonstrated that 8-14 H^+ are released in the extracellular environment as the minimum repeating structure of hydroxyapatite crystals are formed, thus lowering the pH (7-9). To maintain the pH balance, ameloblasts must buffer the protons. During the secretory stage, amelogenins may serve an important role in buffering the pH (9,10); however, during the maturation stage, ameloblasts secrete bicarbonate into the enamel matrix to neutralize the microenvironment (7,9-11). The process of enamel formation requires strict control of extracellular pH (7-9,11); hydroxyapatite crystal growth and proteinase activity in the extracellular

space are pH-dependent phenomena (7,8,12). pH therefore serves an important role during amelogenesis, which is the basis of enamel formation.

The current review discusses the regulation of pH during amelogenesis in dental fluorosis and also explores the effects of changes in ion transporters on dental fluorosis. This may identify future directions of research to identify potential novel treatments of dental fluorosis.

2. Mechanism of dental fluorosis

There are multiple potential causes of dental fluorosis; however, the precise mechanism by which dental fluorosis occurs remain controversial (13-15). Current investigations into these mechanisms primarily focus on the direct effects on the ameloblasts and the indirect effects on the forming matrix (16). Fluoride has three distinct effects on ameloblasts. Firstly, it has two contrasting effects on cell proliferation. Micromolar F^- promotes the proliferation of ameloblasts, while millimolar F^- inhibits their proliferation, suggesting that higher levels of fluoride may inhibit the proliferation of ameloblasts (17,18). Secondly, the differentiation of ameloblasts may be regulated by F^- via the mitogen-activated protein kinase pathway (19). Thirdly, the apoptosis of ameloblasts increases during oxidative stress if F^- levels are high (20). Yang *et al* (21) demonstrated that high levels of fluoride induce the apoptosis of ameloblasts by downregulating Bcl-2. In terms of indirect fluoride-associated effects, fluoride may interfere with the synthesis, secretion and intracellular transportation of enamel matrix proteins in ameloblasts (18,22). The retention of matrix proteins is not only the result of the decreased activity of proteases, including matrix metalloproteinase-20 (MMP-20) (23) and Kallikrein 4 (KLK4) (24), but is also the result of more effective binding to fluoridated apatite (1,13). Furthermore, high levels of fluoride may induce changes the structure and function of amelogenin, which serves an important role in buffering pH, thereby contributing to the disruption of pH regulation (25). pH is important in the mechanisms mentioned above and pH may directly or indirectly regulatet these mechanisms. Duan *et al* (26) identified that residual protein (emdogain) exists in the enamel of patients with cystic fibrosis and dental fluorosis. This was determined to be a consequence of disordered pH levels, which lead to abnormal proteolytic activity and defective endocytosis. Thus, pH serves an important role during the entire process of amelogenesis. It is therefore important to determine how pH is regulated during amelogenesis when intake of fluoride remains high for a prolonged period of time.

3. Amelogenesis and the regulation of pH

There are five stages of amelogenesis, which include: The pre-ameloblast stage, the pre-secretory stage, the secretory stage, the transition stage and the maturation stage (25,27). Of these five stages, two are the most important: The secretory and the maturation stages (4,28). During the secretory stage, ameloblasts secrete a number of proteins, including amelogenin, ameloblastin, enamelin and MMP20 (29,30). These proteins organize the nascent structure of ameloblasts, which are composed of long thin crystal ribbons (29). During the secretory stage, ameloblasts construct the full length of the enamel ribbons; however, this matrix remains only partly mineralized until the maturation stage (29,31,32). During this stage, the extracellular pH is ~7.23 (9,29,33). During the maturation stage, matrix proteins are degraded by a stage-specific protease and crystals develop into their final hardened forms (4,7,28). This stage-specific protease is KLK4, which can degrade matrix proteins and facilitate their resorption (4,34). In addition, high numbers of calcium and phosphate ions are secreted into the enamel matrix (30). This allows enamel ribbons to widen, leading to increased hydrogen release (7,29). During the efflux of calcium and phosphate ions, hydroxyapatite (HA) may be deposited. At the same time with HA deposition, hydrogen ions are released, lowering the pH of the enamel matrix (33). Depending on the phosphate precursor, the precipitation of HA releases 8-14 moles of hydrogen ions per mole of HA, which acidifies the enamel matrix (4,10,29,35). At this stage, extracellular pH may decrease to <6.0 (9,29,33).

However, pH levels may vary during the maturation stage. Due to cyclic oscillations of ameloblasts between the smooth-ended (SE) and ruffle-ended (RE) forms, the pH in the enamel matrix periodically fluctuates between neutral (pH 7.2) and acidic (pH 5.8) (7,11,30,36).

To ensure that pH levels change as required during the different stages, an effective pH regulation mechanism is required. The primary extracellular buffering mechanism used by ameloblasts is the bicarbonate buffer system (7,9-11), particularly during the maturation stage (9,37). During amelogenesis, ameloblasts secrete bicarbonate into the enamel matrix to buffer the protons produced by the growth of hydroxyapatite crystals. During the maturation of enamel, carbonic anhydrase type VI, which is a secreted type of carbonic anhydrase, may catalyze the formation of CO_2 and H_2O from carbonic acid, formed by bicarbonate buffering of the protons in the enamel space (9,38). Furthermore, Sasaki *et al* (11) suggested that an acidic pH in RE ameloblasts may be due to the release of protons, which may contribute to pH regulation. Due to the transportation of protons and bicarbonate, the balance between the intra- and extracellular pH of ameloblasts may be maintained.

4. Electrolyte transport processes involved in pH regulation during amelogenesis

The transport of ions in and out of cells occurs in two ways, active transport and passive diffusion, and this is also the case in ameloblasts. Ion transporters participate in this process. Ions associated with amelogenesis predominantly include calcium, phosphonium, chloridion, protons and bicarbonate, and primarily rely on ion transporters to enter and leave ameloblasts. Previous studies have demonstrated that there are many ion transporters on the membrane of ameloblasts, including Ca_v1, inwardly rectifying potassium channel ($K_{ir}1.1$), epithelial sodium channel, anion exchange protein (AE)1, AE2, electrogenic sodium biocarbonate cotransporter (NBC)1, NBCe1, sodium-calcium exchanger 1-3, sodium-hydrogen antiporter 1 (NHE-1), sodium/potassium/calcium exchanger 4, H^+-adenosine triphosphate (ATP)ase, cystic fibrosis transmembrane conductance regulator (CFTR), H/Cl exchange transporter (ClC)-3, ClC-5, ClC-7, gap junction α-1 protein

(Cx43) and PAT-1 (16,30,36,39-51). Among these ion transporters, some are responsible for regulating pH and include ClC-5, ClC-7, CFTR, NHE-1, NBCe1, AE1, AE2 and pendrin. As aforementioned, the formation of hydroxyapatite during the maturation stage of amelogenesis generates a large quantity of protons; to sustain the growth of crystals, these protons must be neutralized (7,42,52), potentially via the secretion of neutralizing ions, such as bicarbonate (30).

CFTR serves an important role in transporting bicarbonate into the enamel space to buffer protons in ameloblasts and locates on the apical plasma membrane during the maturation stage of ameloblasts (52). Paine *et al* (8) demonstrated that bicarbonate was transported by apical AE2a, basolateral NBCe1 and apical CFTR. Furthermore, previous studies have identified that CFTR is a critical factor in the regulation of pH during the maturation of ameloblasts and is essential for enamel mineralization (52-54). Duan *et al* (26) demonstrated that CFTR inhibition and treatment with CFTR siRNA may increase intracellular pH. Sui *et al* (53) placed incisors taken from mice with the cystic fibrosis gene knocked out in pH indicator solution and indicated that they were acidic. CFTR is a classical Cl^- channel and transports bicarbonate in two main ways, accompanied by the transportation of chloride. CFTR stimulates the transport activity of Slc26a members, leading to bicarbonate efflux (54-56). Additionally, CFTR is permeable to bicarbonate (54,57,58). Although the results of previous studies have indicated that CFTR is more permeable to Cl^- than to bicarbonate, studies have revealed that CFTR may be responsible for >50% of the total bicarbonate efflux in pancreatic duct cells (54,57).

Solute carrier (SLC) 4 bicarbonate transporters serve an important role in the transport of bicarbonate and the regulation of pH in different types of cells (8,59). SLC4A2 codes for AE2, an anion-exchanger; whereas SLC4A4 codes for NBCe1, a bidirectionally electrogenic transmembrane ion-transporter (60). Depending on different cell types, NBCe1 is able to co-transport two or three bicarbonate ions per Na^+ ion (60). However, the location of AE2 and NBCe1 remains controversial. Paine *et al* (8) and Lacruz *et al* (43) indicated that NBCe1 is located on the basolateral membrane and AE2 is located on the apical plasma membrane. However, Bronckers *et al* (61) demonstrated that AE2 is located on the basolateral membrane and that NBCe1 is located in the papillary layer cells of the enamel organ. The difference between these studies may be due to the different methods employed and the different age groups of the animals in each of the studies (49). Overall, it is considered that, following basolateral bicarbonate uptake by NBCe1 and AE2, apical bicarbonate secretion is mediated by CFTR (62). Gawenis *et al* (63) demonstrated that mice lacking AE2 were edentulous (63). Additionally, patients harboring NBCe1 mutations exhibit different levels of enamel abnormalities (64,65). These results all confirm the important role of AE2 and NBCe1 in the transport of bicarbonate in ameloblasts and in regulating pH levels (8).

Pendrin is another member of SLC family, which is encoded by SLC26A4 located on the apical membrane of ameloblasts (45). It is able to transport chloride, bicarbonate, iodine and formate (45). A number of studies have demonstrated that it is able to regulate luminal pH in the kidney, inner ear and thyroid (66-68). However, the transport of bicarbonate by pendrin is not critical for enamel formation. Bronckers *et al* (45) identified that ameloblasts may achieve the normal mineralization of enamel in pendrin knockout rodents.

ClC-5 and ClC-7 are voltage-gated chloride channels, which are responsible for transporting Cl^- and H^+. Previous studies have generally focused on the regulation of dentin development by ClC-5 (41,69). Duan *et al* (70) demonstrated that ClC-5 is also expressed by ameloblasts of tooth germ. The enamel of ClC-5 knockout mice is easily detached from dentin and this may affect enamel formation (41,70). ClC-7 is a Cl^-/ H^+ antiporter (71,72). It has been proven that, during the maturation stage of ameloblasts, the highest levels of ClC-7 are immunolocated in ameloblast vesicles (48,71,72). Osteopetrosis-associated transmembrane protein 1, which centralizes to the lysosomes of all cells and lies on the ruffled border membrane of osteoclasts, is essential for the transport activity of ClC-7 (71-74). In osteoclasts, Kornak *et al* (74) demonstrated that ClC-7 was important for the acidification of the resorption lacuna; however, this was not the case in lysosomes. The lysosomal pH and degree of enamel mineralization did not change following ClC-7 knockout (71,75,76). However, ClC-7 may serve an important role during tooth eruption, as it has been determined that ClC-7 knockout mice experience a failure of tooth eruption (71,74,75,77); however, further studies are required to explore the exact mechanism of action. Thus, unlike its function in osteoclasts, ClC-7 is not critical for ameloblast function (71).

NHE1 is a Na+/H^+ exchanger located on basolateral membrane of ameloblasts and is strongly immunoactivated in the secretory and maturation stages of amelogenesis (42). It co-operates with other transporters to transport H^+ to the enamel matrix, thus altering pH. Carbonic anhydrase (CA) is a zinc metalloenzyme required for the survival of pro- and eukaryotic cells (38,78). It is able to catalyze the reaction of carbon dioxide and water to produce carbonic acid, which then rapidly dissociates into hydrogen and bicarbonate ions (38,78,79). Among >12 CA genes, it has been proven that CA II and CA VI are expressed in maturation ameloblasts (38,78,79). CA II is the most abundantly expressed isozyme in all major mammalian organs and is localized in the cytoplasm. It can pump H^+ into the enamel with the aid of H^+-ATPase type V (80). CA VI is a secreted enzyme located in the enamel, which buffers local pH by providing bicarbonate ions or recycling excess carbonic acid (38,78). Generally speaking, CA II and CA VI participate in the pH homeostasis of ameloblasts by transporting H^+ and HCO_3^-.

5. The effect of fluoride on pH regulation during amelogenesis

Excessive fluoride may cause pH disturbance during amelogenesis. As the pH balance is primarily maintained by electrolyte transporters, fluoride may serve a role during electrolyte transportation. Zheng *et al* (25) demonstrated that fluoride may indirectly regulate ameloblasts in mice and humans. The upregulation of NBCe1 during ameloblast maturation is not directly stimulated by fluoride, as NBCe1 expression is unaffected following the addition of fluoride. The exact mechanism of action of NBCe1 upregulation may

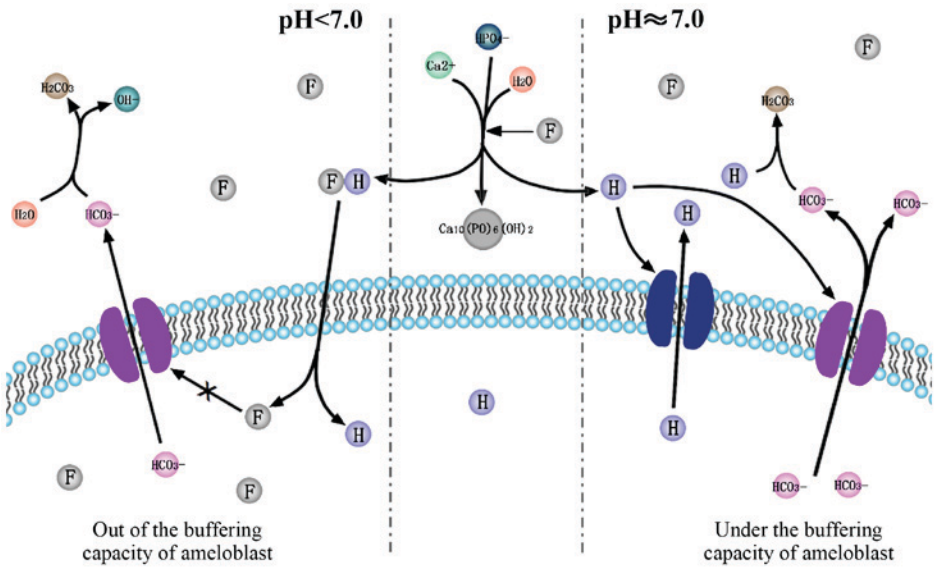

Figure 1. Schematic representation of the association between F^- and pH regulation during amelogenesis. F^- accelerates crystal formation and produces a large number of protons, which may lower the pH of the microenvironment. The decrease in pH upregulates the expression of ion transporters in ameloblasts in two ways: Directly and indirectly, via a currently unknown signaling pathway. These two processes lead to the excessive release of HCO_3^-, which may neutralize the pH to form a microenvironment favoring crystal nucleation. However, when the decrease in pH levels is severe, more F^- diffuses into cytoplasm of amelobalsts along the concentration gradient formed by the release of protons, overwhelming the buffering capacity of ameloblasts. The retention of F^- induces a series of pathological changes, including the dysregulation of ion transportation. The decrease in pH levels cannot be neutralized, which causes the abnormality of crystal formation, leading to dental fluorosis.

be due to mineral deposition and matrix acidification (25). Paine *et al* (8) demonstrated that AE2 and NBCe1 expression is upregulated when pH levels are low. A previous study investigating the association between microRNA (miRNA) 224 expression and acidification indicated that acidification caused by fluoride may downregulate miRNA 224 expression (81). Furthermore, miRNA 224 expression was inversely correlated with the expression of SLC4A4 and CFTR (81). Further studies are required to investigate the direct effect of fluoride on their expression.

The effect of fluoride on pH regulation has been verified. A number of *in vitro* and *in vivo* studies have proven that F^- is able to accelerate crystal formation and induce hypermineralized lines in secretory enamel (6,61,82). The process of crystal growth produces a large number of protons, which can acidify the microenvironment. A decreased pH may induce a series of changes in electrolyte transporters and may also affect the toxicity of F^- (4,8,25,29). F^- cannot enter the ameloblast directly and must be converted to hydrogen fluoride (HF) beforehand (4). A low extracellular pH promotes this conversion; >25 times HF is formed at pH 6.0 compared with at pH 7.4, as determined by the Henderson-Hasselbalch equation (4,29). Due to the concentration gradient of pH, HF can diffuse easily into the cytoplasm from the enamel matrix and revert to F^- in the neutral cytoplasm; it cannot consequently easily diffuse out of the cell (4,29,83,84). Increased F^- concentration in the cytoplasm can induce oxidative stress by reducing the activity of antioxidant enzymes, which affects a variety of structures and processes of normal cells due to reactive oxygen species accumulation (4,85-87). Furthermore, fluoride ions in the cytoplasm may induce endoplasmic reticulum (ER) stress, including the phosphorylation of eukaryotic initiation factor 2, which may result in a decrease of overall protein production, including secretion of the protease KLK4 (4,29,88,89). Thus, F^- induces more severe toxicity in ameloblasts undergoing maturation (90-92).

Based on the acidic hypothesis of F^- and the pH regulation in amelogenesis, the current review hypothesizes that F^- is associated with pH regulation during amelogenesis. F^- accelerates crystal formation in ameloblasts in two different ways (Fig. 1). Firstly, it stimulates the release of protons and lowers the pH of the cell microenvironment. This upregulates the expression of ion transporters, which transport H^+ and HCO_3^-. Yet through this complicated regulation of H^+ and HCO_3^- in and out of ameloblasts by different ion transporters mentioned above, the net efflux of HCO_3^- exceeds H^+. Secondly, it triggers unknown signaling pathways and upregulates bicarbonate transporters, including NBCe1, AE2, CA2, CA6, CFTR. Upregulated bicarbonate transporters release a large amount of bicarbonate from the ameloblasts, which may neutralize the pH to form a microenvironment that favors crystal nucleation. By contrast, following a decrease in the pH, more F^- diffuses into cytoplasm of amelobalsts along the concentration gradient formed by the release of protons. The retention of F^- induces a series of pathological changes, including oxidative and ER stress. Under the buffering capacity of ameloblasts facing F^- toxicity, normal mineralization occurs; however, if the buffering capacity of ameloblasts is overwhelmed by excessive F^-, hypomineralization occurs, which may cause dental fluorosis. Further studies are required to investigate the signaling pathways involved and the exact process by which ions are transported.

6. Conclusions

Amelogenesis is a complicated process that involves crystal formation, the removal of matrix proteins and ions transportation. The pH of the enamel matrix fluctuates during different stages of ameloblast development. Under normal conditions, pH

levels are regulated and ameloblasts may perform their normal function and stimulate normal mineralization. CFTR, AE2, NBCe1, ClC-5, ClC-7, NHE1, CA2, CA6 and H^+-ATPase type V are all involved in the regulation of pH. However, under high fluoride concentrations, pH regulation becomes dysregulated. This causes the malfunction of ameloblasts, resulting in hypomineralization and dental fluorosis. The effect of fluoride on ameloblasts is due to its impact on electrolyte transporters and its direct diffusion into the cytoplasm in an acidic environment.

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Competing interests

The authors declare that they have no competing interests.

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