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FATE OF FLUORIDE-INDUCED SUBAMELOBLASTIC CYSTS IN DEVELOPING HAMSTER MOLAR TOOTH GERMS

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Abstract

White opacities and pits are developmental defects in enamel caused by high intake of fluoride (F) during amelogenesis. We tested the hypothesis that these enamel pits develop at locations where F induces the formation of sub-ameloblastic cysts. We followed the fate of these cysts during molar development over time. Mandibles from hamster pups injected with 20 mg NaF/kg at postnatal day 4 were excised from 1 h after injection till shortly after tooth eruption, 8 days later. Tissues were histologically processed and cysts located and measured. Cysts were formed at early secretory stage and transitional stage of amelogenesis and detected as early 1 h after injection. The number of cysts increased from 1 to almost 4 per molar during the first 16 h post-injection. The size of the cysts was about the same, i.e., $0.46 \pm 0.29 \times 10^6 \mu\text{m}^3$ at 2hr and $0.50 \pm 0.35 \times 10^6 \mu\text{m}^3$ at 16 h post-injection. By detachment of the ameloblasts the forming enamel surface below the cyst was cell-free the first 16 h post-injection. With time new ameloblasts repopulated and covered the enamel surface in the cystic area. Three days after injection all cysts had disappeared and the integrity of the ameloblastic layer restored. After eruption, white opaque areas with intact enamel surface were found occlusally at similar anatomical locations as late secretory stage cysts were seen pre-eruptively. We conclude that at this moderate F dose, the opaque sub-surface defects with intact surface enamel (white spots) are the consequence of the fluoride-induced cystic lesions formed earlier under the late secretory–transitional stage ameloblasts.

Keywords

fluoride; amelogenesis; fluorosis; cystic lesions

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Introduction

High intake of fluoride (F) during stages of dental development results in enamel defects (1–5). The nature and extension of these defects vary depending on F dose, exposure time and a number of other factors including age, calcium homeostasis and bone metabolism, diet- and genetic factors (6–9). Long-term exposure to low F doses gives rise to formation of porous hypomineralized sub-surface enamel below an intensely mineralized enamel surface layer. Clinically this is seen as chalky white opaque areas which are softer than sound enamel (10–12). In more serious cases, extensive opacities consisting of porous sub-surface enamel and local enamel pits are found. How these pits are formed is not clear. Some investigators suggested that they are developed pre-eruptively by a temporary dysfunction of (late secretory) ameloblasts which deposit less enamel causing a local enamel dysplasia (13). Others however suggested that these pits are formed post-eruptively from locally hypomineralized sub-surface lesions that initially were covered by a thin intact enamel surface layer (14). This layer presumably breaks down after eruption due to mechanical usage as teeth become functional.

Several animal studies gave evidence that high plasma fluoride (F) peak levels following injection of F can induce the ameloblastic layer to locally detach from the enamel surface and form a sub-ameloblastic cyst (15–18). The formation of these cystic lesions in rodents occurred principally in developing molar tooth germs but not or only very occasionally in incisors (19–21). In developing rat molars it was reported that all secretory ameloblasts form cysts after a single high dose of F (22); a moderate dose of F administered to hamster pups, affected the cells more selectively depending upon the doses used (23).

Previously we showed that 24 h after injection of fluoride (20 mg NaF/kg body weight or 9 mg F/kg body weight), cyst formation always occurred on top of an intensely hypermineralized enamel surface in early and late secretory stage of amelogenesis (24;25). Late secretory-to-transitional stage ameloblasts are particularly sensitive to F and form cysts after a single dose of 10 mg NaF/kg body weight (4.5 mg F/kg body weight). Early secretory ameloblasts require twice that dose to form cysts. What the consequences are of these cysts for the completion of enamel mineralization and for quality of the mature erupted enamel is not clear. We hypothesize that a cystic lesion under the late secretory-to-transitional stage ameloblasts after administration of a single high F dose will result in a pit (hypoplastic enamel) or a localized hypomineralized porous sub-surface enamel (white spot) seen after eruption. Therefore, in this study, we examined the fate of enamel cysts by following their development from start to end in erupted teeth. For this study, neonatal hamster pups were administered a single 20 mg NaF/kg body weight, a fluoride dose known to induce cyst formation both in some populations of early as well as late secretory-to-transitional stage ameloblasts (26). This dose enabled us to compare the fate of cysts at both locations at the same time.

Materials and methods

Animals

Hamster pups (a total of 26 pups, four to five days old and weighing between four and five grams) were administered one intraperitoneal injection of 20 mg NaF/kg body weight (9 mg F/kg; dissolved in 20 µl distilled water/g body weight). Control pups of the same age (total of 19) received equimolar levels of Cl (as NaCl). Procedures were approved by and performed according to guidelines of the Committee for Animal Care (DEC) of the Free University.

Histological processing

After 1, 2, 16, 24 and 76 h post-injection pups (group sizes of three to five pups, 22 pups; 15 controls; were weighed again, sacrificed and the mandibles excised. The mandibles from the 1 – 24 hr groups were fixed in 5% paraformaldehyde in 0.1 M phosphate buffer with 5% sucrose for 24 h, rinsed in buffer and embedded undecalcified in Historesin® (Leica, Microsystems, Mannheim, Germany). The mandibles from the 76 hr group were decalcified after fixation in 5% buffered EDTA for three weeks and also embedded in Historesin. Serial sagittal sections 5 microns thick were made with stainless steel knives through the entire first and second molars using a Reichert-Jung K (Leica, Microsystems, Mannheim, Germany) heavy duty microtome. An average of 80 glass slides were collected per hemimandible, each slide containing 4 tissue sections. Serial sections of a total of 37 hemimandibles from 22 experimental pups and 15 control pups was collected and analysed by histomorphometry. Initially every tenth slide was stained with hematoxylin eosin (HE) according to Mayer and every eleventh with toluidine blue in (0.1%) borax.

Morphometry

By screening of every tenth section (stained with HE) the approximate position of each cyst could be determined. For a complete three dimensional outline of the cysts, more sections were stained (every second or third glass slide) till the entire cyst was covered from begin to end. Then the surface area of each cyst were measured at 100x magnification using a computerized Leica DMBLM microscope equipped with a digital camera and QWin v3 software (Leica, Microsystems, Mannheim, Germany). From each cyst the outline was traced by hand (from distal membranes of the detached ameloblasts till the enamel surface). The measured data were stored in an Excel program. The entire stack of measured surface areas was summed per cyst, the sections in between calculated by extrapolation from the measured sections, multiplied with the number of sections and thickness of each section to give a final approximate volume of each cyst. The volumetric values of all cysts are presented as mean and standard deviation for each treatment group. Student t test (two-tailed, unpaired) was used to calculate statistical significance between control and experimental groups at $p < 0.05$.

Lasting effects in post-eruptive stages

To examine the effect of cysts on the final quality of enamel, the molars of four experimental and four control hamster pups injected with respectively 20 mg NaF per kg body weight or an equimolar amount of Cl (as NaCl) and allowed to erupt into the oral

cavity (Table 1). Pups were sacrificed at post-natal day 12 (i.e., eight days after fluoride injection). To minimize the chance that hypomineralized areas with intact surface formed pits by damage of the surface layer post-eruptively due to functional use, the teeth were collected immediately after eruption while pups were still weaning. Mandibles and maxillae were fixed as afore mentioned and stored in 70% ethanol. Changes in smoothness of the enamel surface were examined at 10x magnification using a stereo microscope. The tooth enamel surface was examined moist to locate any surface irregularities, pits or depressions. Then, the enamel surface was air-dried and again inspected also at 10x magnification to reveal the typical white opacities or pits. The number of opacities and/pits were scored in one mandibular and one maxillary first molar from each hamster.

Results

The injections with F did not change the gain in body weight when compared to control pups injected with Cl. At time of fluoride administration, the first mandibular molars were in the maturation phase in coronal portion near the cusp tips whereas some proliferation and differentiation of new ameloblasts still occurred at the crown base at the cervical loop. Serial sections showed that cysts were present only in developing first molars but not in second molars that had not yet started enamel formation at time of injection. In first molars of the F group, cysts were located basically at two different sites: near the cervical loop area where newly differentiated secretory ameloblasts had just deposited the initial layers of enamel and more occlusally, in late secretory-transitional ameloblasts, about the 1/3 to 1/2 the cusp height down from the tip (Fig 1).

After F injection, by histology, 48 cysts were found in the 22 molars analysed, 27 cysts in early secretory stage and 21 cysts in late secretory/transitional stage. The average number of cysts detected per molar increased from one cyst at one hour post-injection to almost four cysts at 16 h (Fig 2). After 24 h, the number was slightly lower than at 16 h (Fig 2). Surprisingly, three days after injection, no cysts could be found any more. At that time, the entire surface of enamel was covered with a well-organized layer of functional ameloblasts. None of the 15 control pups injected with Cl and analysed by histology contained cysts.

The total volume of the cysts (early and late secretory stage together) did not change the first 24 h after injection (Fig. 3) and measured between 0.4 – 0.5 mm³/cyst. When examined separately at both locations, the volume of the cysts was the same (Fig. 4). All cysts were gone three days after injection.

Eight days after injection four experimental and four control animals were sacrificed and mandibles and maxillae excised for visual inspection of erupted molars at 10x magnification. First molars had just erupted, second molars not yet or their cusp tips had just started to erupt. In the first molars loose shreds of brown reduced enamel organ were still present in the fissures or in cervical portion of the crowns suggesting eruption had just occurred or still occurring. After air-drying, white opacities became apparent located at about 1/3 to 1/2 of the height of the cusp (Fig 5). The boundaries of the opacities were generally diffuse. Each molar contained at least one white spot; a total of 21 white spots were found in the eight fluoride-treated molars examined. Two thirds of these white spots

(14 white spots) were located on the lingual side in both maxillary and mandibular first molars. This number was about half of the total number of cysts per molar. Molars from animals injected with NaCl had no opacities.

Discussion

The present data show that cysts formed in the ameloblastic layer after a moderate exposure to F can give rise to white opaque areas that become apparent when the teeth erupt. The approximate location of these white areas (1/3 to 1/2 from the cusp tip) closely matches the position and the number of the coronal cysts seen in pre-eruptive stages representing the position of the damaged late secretory/transitional stages due to fluoride administration. Hence, one mechanism by which white opaque areas are formed in fluorotic enamel is by cyst formation at locations of late secretory/transitional ameloblasts. The opaque areas formed at this dose of F had an intact enamel surface. It is conceivable that these opaque white spots may develop into pits in the oral cavity consistent with the concept that some of the pits found in human fluorotic enamel can be formed post-eruptively by loss of the thin surface layer covering a porous subsurface lesion (27).

Remarkably, some recovery of the enamel lesion can take place pre-eruptively, as illustrated by the disappearance of the cysts three days after injection; a phenomenon also reported earlier in developing rat molars (28). New layers of ameloblasts presumably migrated coronally to repopulate the cell-free surface of the enamel below the cysts. Whether this action required an expansion of the population ameloblasts by proliferation and differentiation down at the cervical loop region or by rearrangement and/or volumetric change of the existing ameloblasts to fill the cell-free gap along on the enamel surface is not clear. This suggests a high plasticity of ameloblasts to migrate, form a new coherent ameloblast layer and cover cell-free fluorotic enamel from which ameloblasts have detached earlier. The opacities found in erupted fluorotic enamel contained a smooth apparently well-mineralized surface layer. Collectively these data suggest that 'secondary' ameloblasts apparently are quite successful in partly restoring the enamel lesion below the fluoride-induced cysts.

Cysts were also formed at early secretory stage but there was no clear sign of white opacities at the cervical part of the crown after eruption. In organ culture, we found that removing F from the culture medium, young secretory ameloblasts completely recovered and resumed deposition of new layers of enamel (29). This conceivably also occurs *in vivo* as long as proliferating cells in cervical loop region can generate new ameloblasts that migrate occlusally. Damage to the initial layers of enamel consequently will be repaired by deposition of new layers of sound enamel which deeply bury the early defects. This probably explains why post-eruptively, no white spots were located in cervical areas of the crowns. Deep cervical lesions have been reported in seriously fluorotic erupted teeth (12;19;20). Such cervical pits may have been formed in teeth in which crown size had already been completely outlined in which no new ameloblast were generated after the death of early secretory ameloblasts.

In conclusion, sub-ameloblastic cysts formed by injection of moderate levels of F give rise to porous hypomineralized enamel with intact surface at locations where the ameloblasts were in the late secretory-transitional stage at the time of fluoride administration. Due to mechanical loading of the molars after eruption, such areas are likely to develop into shallow enamel surface pits.

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Fig. 1.

Microphotographs of developing first mandibular molar from a hamster pup injected with a single dose of 20 mg NaF/kg (9 mg F/kg) body weight. Two types of cysts have developed: cervically (thick arrow, inset) near early secretory ameloblasts and occlusally (double arrow, inset) below late secretory/transitional ameloblasts. The transitional ameloblasts (tA) surrounding the cystic lesion are highly disorganised (arrows). Numerous sectioning artifacts (asterisks) are unavoidable due to the hypermineralization of the cystic enamel surface (arrowheads). The white double arrow shows the position of the characteristic fluoride-induced double response line in the enamel (E) HE staining. sA: secretory ameloblasts; SR: stellate reticulum; SI: stratum intermedium; DP: dental pulp; D: dentin; O: odontoblasts; pd: predentin; B: alveolar bone (Bar = 100µ; inset: 200µm).

Fig. 2.

The number of cysts per first mandibular molar found after injection of 20 mg NaF/kg body weight varies over time. Bars represent the average number of cysts per molar (early secretory and late secretory stages combined). Mean and standard deviation. Numbers in parentheses indicate number of pups examined. Control pups injected with NaCl did not contain cysts. Note that 76 h after injection cysts have completely disappeared. * $p < 0.05$; F vs. Cl.

Fig. 3. Average volume of the cysts (μm^3) measured at various times after injection of 20 mg NaF/kg. Bars represent volume per cyst (early secretory and transitional stage combined). Means and standard deviation. Note that cysts were not found at 76 hours after injection.

Fig. 4. Average volume of early ('early' or cervically located) and late ('late', coronally located) secretory cysts (μm³) separately measured at various times after injection of 20 mg NaF/kg. Means and standard deviation. Numbers indicate number of cysts measured. Note that 76 h after injection both cervical and occlusal cysts have disappeared.

Fig. 5.

Photomicrograph of a newly erupted first molar (day 12 after birth) with two white opaque areas (circled) with an intact enamel surface seen after air-drying of the tooth before examination. Pups were injected with a single dose of 20 mg NaF/kg body weight at day four postnatal and sacrificed eight days later to evaluate the effect of the pre-eruptive fluoride administration on the erupted first molars. Note that the lesions are located half way to one third to the cusp tips in occlusal area corresponding to the position of the late secretory-to-transitional stage ameloblasts at the time of fluoride injection. None of the molars of the pups injected with Cl^- contained white opaque areas or pits.

Post-eruptive analysis of white spot formation in erupted mandibular and maxillary first molars.

Table 1

Post-injection Time	F injection *			CI injection *		
	number pups	number spots		number of pups	number of white spots	
		cervical	occlusal		cervical	occlusal
8 days **	4	0	21	4	0	0

* pups received a single injection of 20 mg NaF/kg b.w. or NaCl at day 4, t=0

** Post-injection “Day 0” = neonatal day 4.