THE EFFECTS OF FLUORIDE ON VARIOUS STAGES OF ENAMEL FORMATION IN VITRO

J.H.M. WöLTGENS and D.M. LYARUU

Laboratory of Tooth Development, Dept. of Oral Cell Biology
ACTA, Vrije Universiteit, de Boelelaan 1115
1081 HV Amsterdam (The Netherlands)

SUMMARY

A survey has been given on the effects of various F^- concentrations on different stages of amelogenesis in vitro. For short-term-exposure the transitional ameloblast seems to be very sensitive (4-6 µMol/L F^-).

52 µMol F^- (1ppm) inhibited mineralization of newly formed matrix in favour of hypermineralization of already existing crystallites. This matrix re-started to mineralization after removing the F^-.

No direct histological in vitro effects of F^- on the maturing ameloblasts were demonstrated. These in vitro effects which may also be reached in the plasma of children after F treatment are used to explain the clinical appearance of fluorosis.

INTRODUCTION

Overwhelming literature is available on the caries reducing effects of fluoride (F^-) [1]. However, less attention has been paid to the risk of the inappropriate use of F^- on enamel formation. In this respect even the combined use of F^- tablets and tooth brushing with a F^-containing toothpaste (0.1% F^-) twice a day by young children up to 4 years can lead to unacceptably high

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plasma F^- concentrations in which mottling of the forming enamel can occur, because 25% of the toothpaste is swallowed by children younger then 4 years of age. Unfortunately, very little is known about the development of mottled enamel, the stages at which the ameloblast is sensitive for excess of F^- [2] or what the minimum F^--concentrations are at which the ameloblast can directly be affected.

In order to get more insight in these questions we developed an organ culture system of tooth germs in which it was possible to get normal enamel formation and to measure the direct effects of F^- on the developing tooth.

MATERIALS AND METHODS

Teeth: For the long-term in vitro experiments, non-mineralizing second (M^2) maxillary molars of 3 and 4 day-old-hamsters (Mesocricetus auratus, L) were used. The ameloblasts are in the secretory and transitional phase from day 4 to day 6 of cultivation.

For the short term and recovery in vitro experiments first M^1 molars of 3 day old hamsters were used.

Dissection procedures

The tooth germs were aseptically dissected from animals killed by decapitation. The germs were maintained in plastic Petri-dishes containing 3.10 ml prewarmed culture (BGJ_b) medium. This medium contained 15 per cent fetal calf serum and was continuously flushed at 37°C with a gas mixture consisting of 45 per cent N_2, 50 per cent O_2 and 5 per cent CO_2 until all the dissections were completed. The average length of this dissection period was 2 h.

The contralateral tooth germs used for controls were cultured in the same medium supplemented with an equimolar amount of Cl^-.

Culture procedures

Long-term experiments: Second maxillary molars of 3-4 day-old hamsters were cultured for up to 8 days in the continuous presence of F^- or chloride (Cl^-) in concentrations between 2.63 µM and 1.31 mM. For biochemical study, explants were labelled during the last 24 h of culture with a triple label of [^{3}H]-proline, ^{45}Ca and $^{32}PO_4$. The ^{3}H-labelled amelogenins were separated from the ^{3}H-labelled dentine collagens by a three-step extraction procedure. The proline-labelled amelogenins were isolated by sequential extraction with water and formic acid and their nature examined by proline SDS-PAGE and collagenase digestion [3]. The results were compared with histological and electron microscopical findings.

Short-term experiments: The effect of various concentrations of F^- on cell proliferation, matrix formation, resorption and mineralization was examined in hamster molar tooth germs in premineralizing and mineralizing stages. The exposure lasted 16 h (mineralizing stages) and 24 h (premineralizing stages) and the F^- levels ranged from 2.63 µM to 2.63 mM; [^{3}H]--thymidine [^{3}H]-proline, ^{45}Ca and $^{32}PO_4$ were used as markers for cell proliferation, matrix formation and mineralization, respectively. The recovery of mineralization capacity of fluorotic enamel matrix was investigated in 3 day old hamster first molar tooth germs already pre-exposed in organ culture to F^- for 24 h during the secretory phase. The germs were then cultured for another 24 h in a fresh medium without F^-. The results were compared with studies by light- and electron microscopy.

Histology

The cultured germs were fixed with intermittent agitation for 1-2 h in 3 per cent glutaraldehyde in 0.1 M sodium-cacodylate buffer, pH 7.35, containing 5 per cent sucrose and 0.01 per cent OsO_4. The germs were then rinsed with intermittent agitation in the buffer containing 5 per cent sucrose for at least 1 h at room

temperature. Post-fixation was done in 1 per cent OsO_4 in 0.1 M sodium-cacodylate buffer, pH 7.35 for 1 h at 0-4°C. After a brief rinse in distilled water, the tooth germs were dehydrated through a graded ethyl-alcohol series, cleared in propylene oxide and embedded in Spurr low-viscosity resin [5].

One half micron semi-thin sections were cut with glass knives in a bucco-lingual plane approximately through the middle of the anterior cusps and stained in 0.1 per cent toluidine blue in 0.1 per cent sodium tetraborate solution. The cervical-loop region containing both pre-secretory and secretory ameloblasts was trimmed off and sectioned for electron microscopy using a diamond knife. The ultra-thin sections (~70 nm) were observed in a Philips EM 301 electron microscope, initially unstained and then stained in 2 per cent uranyl acetate in distilled water. The electron microscopy findings were compared with those obtained by light microscopy.

RESULTS

Figure 1 gives schematically the various stages of the ameloblasts during their development. The lowest effect of F^- directly from the medium or from F^- incorporated earlier in the tooth germs on the consecutive stages of the ameloblasts, pre-

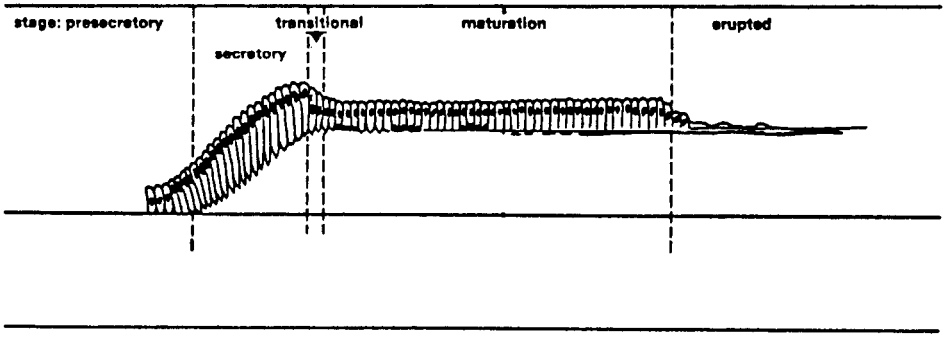

Fig. 1. Schematic representation of the life cycle of ameloblasts.

TABLE 1

Survey of the lowest F^- concentration which influences every stage of enamel development in vitro

Stage of development	F^- treatment			
	Short-term parameter (1-2 days)		Long-term parameter (4-8 days)	
a. pre-ameloblasts	1.3 mM	histology	1.3mM	histology
b. presecretory and early secretory ameloblasts	52 µM	histology	1-2 µM	biochemistry
c. secretory ameloblasts	52 µM	biochemistry (mineralization, matrix breakdown)	-	-
d. transitional and early maturation stage*	4-6 µM	biochemistry (mineralization)	-	-
e. late maturing ameloblasts	1.3 mM	histology	1.3 mM	matrix breakdown

* preliminary data

ameloblasts, presecretory and (early) secretory ameloblasts, the secretory ameloblasts, the transitional and early as well as the late maturing ameloblasts are given, both for short-term (1-2 days) and long-term (4-8 days) F^- treatment.

From this scheme it can be derived that during long-term treatment the secretion of extracellular amelogenins is already inhibited by 1-2 µMol F^- [6]. This concentration is very close to the minimum concentration of 2 µMol/L in the plasma by which fluorosis can be obtained during chronic ingestion [7].

Preliminary results suggest that for short treatment with F^-- during cultivation, the transitional ameloblasts seem to be very sensitive (4-6 µMol)[8]. Finally during short-term treatment of the secretory ameloblast in the mineralizing first molar with 1 ppm F^- new matrix was formed which did not mineralize. Instead of this, hypermineralization of the already existing crystallites was found. By culturing the F^- treated teeth in a F^- free medium for another day the non calcified matrix again started to mineralize [9]. No direct histological effects of F^- could be found on the maturation phase in vitro.

DISCUSSION

From all effects of F^- on short-term or long-term culture, we have given only the effects by those F^- concentrations, which normally can be found in the plasma (2-10 µMol/L) in areas with optimal fluoridation of 1 ppm or after the use of F^- containing toothpastes, F^- application, or F^- rinsing in children with developing teeth [7].

With these in vitro experiments we now can interpret the clinical findings in developing enamel. It has been reported that teeth of animals treated with F^- are smaller [10]. This phenomenon can be explained by our long-term experiments in which it has been found that even at F^- concentrations below 10 µMol/L the secretion of the enamel proteins as well the Ca^{2+} uptake in vitro can be inhibited [6] leading to thinner enamel and consequently

smaller tooth crowns. In this way also the smaller mesiodistal distance of the toothcrown as found by Kruger [10] could be explained.

In another long-term in vitro study it was shown that the secretion of enamel proteins are regulated by Ca^{2+} concentrations [11]. The inhibition of the enamel proteins during the secretion phase can now be explained by a lower Ca^{2+} uptake in the enamel organ, leading to a diminished secretion of enamel proteins. Also the dramatic effects of low Ca^{2+} concentrations in the serum on the enamel formation, as has been demonstrated by Nikiforuk and Fraser [12], points towards a fundamental role of Ca^{2+} in the enamel formation. At concentrations below 2 µMol/L F^- in the medium during long-term experiments no effects on transitional or maturing amelobasts could be found. This explains why at these concentrations in the plasma teeth have a normal appearance without mottled enamel. However, at concentration of F^- from 5 µMol/L on, even during short-term in vitro experiments the transitional amelobasts seem to be very sensitive when the full thickness of the enamel is being formed. The affected cells appear to be responsible for the more porous enamel surface, as observed later after eruption, because in vitro no direct F^- effect could be found on the maturation even under long-term exposure at higher concentrations of F^- in vitro.

Finally it should be mentioned that short-term exposure of mineralizing tooth germs to 1-5 ppm F^- in vitro resulted in the formation of a new matrix, which did not mineralize, together with a hypermineralization of the enamel already mineralized before the F^- treatment [13]. With respect to its electrophoretic behavior and phosphorylation, the unmineralized matrix was not different from the mineralized matrix [14]. Moreover during cultivation of the tooth germs in F^- free medium, the matrix retained its capacity - although to a lower extent - to mineralize even after this unphysiologically high F^- exposure. The hypermineralization of the enamel was not reversible [9]. This means that the F^- effects on mineralization during the secretory phase are reversible, in such a way that during this phase of tooth development the tooth can recover from a plasma F^- peak. The hypermineralized enamel remains behind in the tooth as characteristic bands in fluorotic enamel.

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