Deborah Jeffrey

From:	Dr John Ryan <[redacted]
Sent:	Friday, 9 November 2012 3:46 PM
To:	Finance and Administration Committee
Subject:	FW: Submission re Ammendment to Water Act Clause 82
Attachments:	Submission Paper Water Fluoridation Amendments.docx; Chronic Fluoride Toxicity Dental Fluoroisis DenBensten 2011.pdf; Tooth Eruption Primary Teeth.pdf; EPA IRISFluorides, Hydrogen Fluoride and Fluorine p 255-263.pdf; Effect of F on Developing tooth Robinson et al 2004.pdf; Fluoride levels in Human Plasma and Breast Milk.pdf; INFANT_FORMULA_CLIFFORD_2010.pdf; Longitudinal Study of Australian Children 2011.pdf; Molecular Mechanism of Action of Fluoride on Bone Cells.pdf; Nutrient Reference Values for Australia and New Zealand.pdf; The Science and Practice of Caries Prevention JADA 2000.pdf; Tooth Eruption Primary Teeth.pdf

Subject: Submission re Ammendment to Water Act Clause 82

The Research Director
Finance and Administration Committee
Parliament House
George Street
BRISBANE QLD 4000

Dear Sir/ Madam
Please find attached my submission re Clause 82 of the Amendment of s8 relating to fluoride in the water supply
Yours Sincerely,
Dr John Ryan
[redacted]

Submission Paper

Dr John Ryan
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6/11/2012

Dr John Ryan: MBBS (Qld,1971), M. Sc. Nutrition, Distinction (Lon.1980), FRACGP, DCH (RCP&S,Irel), MRCGP, FACNEM (Nutritional& Environment. Med) Dr Ryan was a member of the Complementary Evaluation Committee (CMEC) of the Therapeutic Goods Administration (TGA for five years, twice being reappointed.

The Amendment to the Water Act

Clause 82 Amendment of s 8 (Exemption from requirement to add fluoride to relevant public potable water supply)

(1) Section 8(1), 'A public potable water supplier for a relevant public potable water supply may apply in writing to the Minister for an exemption from the requirement under section 7'—

Please amend as follows:

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1. **Reduction of Fluoridated water levels to levels suggested by the US Environmental Protection Agency (USEPA) of 0.06 mg fluoride/kg/ day**

   This is the estimate of daily exposure that is likely to cause appreciable degrees of dental fluorosis during a lifetime.

   Dental fluorosis is a recognised complication of excess fluoride in the first months of life. Fluoride acts topically (11); therefore the inclusion of any fluorides before tooth eruption should at least follow NHMRC (2005) guideline recommendations which are 0.01mg per day for infants 0-6 months and 0.50mg per day from 7 to 12 months. Enamel fluorosis is associated with cumulative fluoride intake during enamel development, but the severity of the condition depends on the dose, duration, and timing of fluoride intake. The effect of fluoride on the dentition is dose-dependent and is not confined to increased caries resistance but has effects on many enzyme systems including the fluoride-sensitive osteoblasts (1). Enamel fluorosis and primary dentin fluorosis can only occur when teeth are forming (2). The transition and early maturation stages of enamel development appear to be most susceptible to the effects of fluoride (3, 4). The eruption of deciduous teeth does not occur until after 6 months of age (5).

Intake of baby formula varies. And while the largest intake of fluoride was found at four months of age, the two week old infants receive the greatest amount of fluoride relative to their body weight (6).
Fluoride levels in prepared formula products should be kept at least to the NHMRC (2005) guidelines with very minimal fluoride allowance for the first 6 months and minor amounts in the first 2 years . Recommendations for exposure suggested by the US Environmental Protection Agency (USEPA) is 0.06 mg fluoride/kg/ day, which is the estimate of daily exposure that is likely to occur without any appreciable risk of deleterious effects (any degrees of dental fluorosis) during a lifetime (7). In the review by Cressey et al (8) and Clifford (9) and the mean levels of fluoride in formula contains 0. 49ug/F and 0.76mg/L respectively, and the exposure for the first 6 months of life is 0.11mg/kg body weight which exceeds all recommendations and greatly exceeds breast milk (10, 14).

Anderson et al (2004) estimated the acute and chronic levels of exposure to infants from birth to 4 months fed with formula made from fluoridated areas in Ireland (6). The exposure was examined for fluoride intake per body weight. Two week old infants were shown to have the greatest intake relative to their body weight. The chronic exposure levels were estimated to be 0.106-0.170mg/kg body weight per day which are in excess of acceptable levels mentioned above.
While susceptibility to fluorosis is greater in the first year of life (Bardsen, 1998), the findings indicate that early mineralising teeth are highly susceptible to dental fluorosis if exposed to fluoride from the first and to a lesser extent the second year of life (4). Fluoride retention rates in pre-eruptive teeth as well as bone are in the order of 85% for infants in comparison with around 50% in adults with increases the likelihood of fluorosis in infants.
Riordan (2002) recorded the prevalence of dental fluorosis of 10 year old children in Western Australia and overall 18.2% of participants had some degree of dental fluorosis. Prevalence for residents in the fluoridated area was 20.68% and 15.1% in nonfluoridated areas. With a statistically significance of 21.9% versus 11.6% however there was no statistical difference in caries rates (15).

2. Water authorities to clearly add a warning for infants to all households receiving fluoridated water

Reasons for this clause relates to the infant fluoride intake that exceeds all medical and dental recommendations as outlined above. While breast feeding is preferred; non breast fed infants rates in Australia vary in the first 6 months with 71% being breast fed at one month decreasing to 14% at 6 months (13). Consumption of formulas with mean fluoride levels up to 0.49ug/g as well as the addition of fluoridated water between 0.7 and 1.2mg/L will greatly enhance the chances for fluorosis in approximately 75% of babies at age 6 month. (See attachments)

3. Exclusion of fluoridation plants in areas of high lead and aluminium

(research on this issue to be e-ailed separetly)

Reasons: Studies have shown links to both higher fluorosis and neurological disorders associated with fluoride, aluminium and lead.

There is much concern for areas with mining of metals such as lead and aluminum (16). Both of these are in the air and on the ground and levels in mining communities have enormous difficulty in keeping the population safe. These cause neurological harm at very small levels because their affect on many enzyme systems (17, 19, 20).

While fluorosis is a recognised side effect of excessive fluoride intake there are also other concerns. Water authorities are encouraged to reduce aluminium in drinking water levels to 0.1mg/L. The average daily ingestion of aluminium from infant formulas for a child of 6 months varied from 200ug to 600 µg of aluminum (18). For this reason the aluminium content of infant formula should resemble that of breast milk which is between 15-30ug/L.

Leite et al (2011) found an exacerbation of fluorosis in rats co-exposed to lead (21). Australia's mining towns with high blood lead levels need extra care from environmental and health caretakers specifically in areas that already have problems with mental effects from high environmental lead.

The above matters are of concern and these concerns should be reflected by water authorities.
Please accept the above submission.

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Caries Research

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The Effect of Fluoride on the Developing Tooth

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Key Words
Apatite · Cell structure · Enamel · Fluoride · Fluorosis · Proteases · Protein-matrix

Abstract
This review aims to outline the effects of fluoride on the biological processes involved in the formation of tooth tissues, particularly dental enamel. Attention has been focused on mechanisms which, if compromised, could give rise to dental fluorosis. The literature is extensive and often confusing but a much clearer picture is emerging based on recent more detailed knowledge of odontogenesis. Opacity, characteristic of fluorotic enamel, results from incomplete apatite crystal growth. How this occurs is suggested by other changes brought about by fluoride. Matrix proteins, associated with the mineral phase, normally degraded and removed to permit final crystal growth, are to some extent retained in fluorotic tissue. Fluoride and magnesium concentrations increase while carbonate is reduced. Crystal surface morphology at the nano-scale is altered and functional ameloblast morphology at the maturation stage also changes. Fluoride incorporation into enamel apatite produces more stable crystals. Local supersaturation levels with regard to the fluoridated mineral will also be elevated facilitating crystal growth. Such changes in crystal chemistry and morphology, involving stronger ionic and hydrogen bonds, also lead to greater binding of modulating matrix proteins and proteolytic enzymes. This results in reduced degradation and enhanced retention of protein components in mature tissue. This is most likely responsible for porous fluorotic tissue, since matrix protein removal is necessary for unimpaired crystal growth. To resolve the outstanding problems of the role of cell changes and the precise reasons for protein retention more detailed studies will be required of alterations to cell function, effect on specific protein species and the nano-chemistry of the apatite crystal surfaces.

Fluoride ion has played a major role in dramatically reducing dental caries over the past 40 years. The discovery was made by comparing caries incidence in individuals exposed to so-called high-fluoride water supplies with that in individuals exposed to lower levels [Dean et al., 1942]. It was deduced from these data that fluoride exposure during tooth development was a prime cause of caries reduction. Since teeth from high-fluoride areas had accumulated higher concentrations of fluoride compared with those from low-fluoride areas, fluoride content of the dental tissues was cited as a major factor in reduced caries incidence.

The effect of fluoride on the dentition is dose-dependent and is not confined to increased caries resistance. Above certain levels in the water supply, visible changes to the teeth, particularly the enamel, become evident. This is the condition known as dental fluorosis.

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Visible Effects of Fluoride on Dental Enamel

The effects of fluoride on dental enamel are well documented [Dean and Elvolve, 1937; Fejerskov et al., 1977; Thylstrup and Fejerskov, 1978]. At about 1 ppm fluoride (53 μM) in the water supply, visible signs of fluorosis begin to become obvious on the enamel surface as opacities, implying some porosity in the tissue. As dose increases, these become more obvious until at 10 ppm (530 μM) or so, the porosity is such that the enamel is physically compromised and large pieces may be fractured from the tooth especially after eruption. The porosity appears to derive from incomplete crystal growth such that the normal close juxtaposition and interlocking of crystals does not occur.

Selective Effect of Fluoride on the Mineralised Tissues

The reasons for the apparent selective effect of fluoride on the skeletal and dental tissues and enamel in particular have been related to the interaction between fluoride ions and the skeletal mineral, calcium hydroxyapatite, dealt with in detail below.

Fluoride is the most electronegative of the elements and is of small ionic diameter. Its resulting high charge density endows it with a great capacity to form strong ionic and hydrogen bonds. This provides the fluoride ion with a potential for interacting both with mineral phases and organic macromolecules. Because of these properties, particularly its small size, it can also act as a 'structure former' in water. This can decrease the mobility of water molecules in solution and in hydration layers of proteins and apatite surfaces with concomitant effects on ligand binding and exchange.

Interactions with the mineral phase have two kinds of effect. First a direct effect on the properties of the mineral itself and its relationship with the enveloping and modulating extracellular organic matrix. Second, the selective concentration of fluoride at the surfaces of mineralised tissues [Robinson et al., 1996] may give rise to elevated fluoride in the immediate vicinity of mineralised tissue cells such that local concentrations may be much higher than those of the tissue fluids in general.

Information on local fluoride concentrations in tissue fluids is limited, however, especially in the immediate neighbourhood of fluoridated apatite. In the enormous amount of work which has been carried out the actual concentrations responsible for any given effect are perhaps the most difficult of areas to clarify. Plasma fluoride concentrations seem to be of particular relevance, relating closely to dietary supply and being around 1 μM at 53 μM in the water supply rising to about 4 μM at 265 μM [Guy et al., 1976; Speirs, 1986]. Wherever possible, therefore, plasma concentrations or those in the immediate tissue environment such as enamel fluid or culture medium have been quoted.

Site(s) of Action of the Fluoride Ion during Odontogenesis

While the effects of fluoride on odontogenesis are well established, the precise site(s), stage(s) of development, timing, and mechanism of action are still unclear. The most likely sites are: (a) cells of the tooth-forming tissues: proliferation, differentiation, functional morphology; (b) extracellular matrix of tooth tissues: matrix protein synthesis secretion, processing and loss; (c) mineral phase: initiation, crystal growth, chemical properties, and (d) extracellular matrix-mineral interactions in tooth tissues.

Effect of Fluoride on Odontogenic Cells

Stage of Fluoride Uptake

In enamel, long considered to be the most susceptible of the dental tissues, fluoride accumulates throughout the developing tissue but especially at its surface. This occurs selectively both very early in amelogenesis and later, across the transition/maturation stage border [Weatherell et al., 1975, 1977]. At this late stage, full tissue thickness has been achieved; the supporting extracellular matrix largely replaced by fluid and considerable growth in crystal thickness begins. Selective uptake may thus be due to the highly porous, hydrated nature of this developmental stage [Hiller et al., 1975; Robinson et al., 1981, 1988]. Substantial amounts of fluoride are then lost during subsequent maturation. This implies that much of this fluoride may be labile and together with the reported lowering of pH at this stage, which would dissolve mineral surfaces [Sasaki et al., 1991], locally elevated fluoride concentrations are likely. Cells associated with matrix withdrawal and crystal growth during maturation could thus be exposed to locally high fluoride concentrations. Suggestions that fluorosis can be induced by elevating concentrations only at this latter stage are consistent with these data [Richards et al., 1986]. While efforts to determine fluoride concentrations in enamel fluid have been made [1 μM, Aoba and Moreno, 1987], this did not distinguish

between developmental stages and must be regarded as an average.

Cell Proliferation

Work with tissue culture using 'pre-ameloblasts' has so far revealed no alterations in DNA synthesis at fluoride concentrations up to 1.31 μ*M* [Bronckers and Wöltgens, 1985] or in frequency of mitotic figures at concentrations up to 1.06 m*M* [Lyaruu et al., 1986].

While the effect of fluoride on proliferating odontogenic cells is equivocal, it is worth noting that bone cells in culture have shown sustained mitosis in response to fluoride [Wergedal et al., 1988 (20 μ*M*); Khokher and Dandona, 1990 (>250 μ*M*)]. This was attributed to intracellular signalling pathways associated with mitotic activity. Inhibition by fluoride, of tyrosine phosphorylase phosphatase, part of the mitogen-activating protein kinase (MAPK) system, has received particular attention [Lau and Baylink,1990] together with activation of G proteins which stimulate protein kinase C. Inhibition of this phosphatase would tend to sustain mitotic activity by maintaining levels of active tyrosine phosphorylase, a mediator of mitotic activity. Why ameloblasts have not shown increased mitosis is not clear. The effect indicated may be specific to bone cells. However, dividing ameloblasts may already be near maximum 'mitotic activity' during tooth formation and any increase over such high activity may not be discernible. Such modest enhancement of mitosis might, however, be in part responsible for alterations to tooth size and morphology attributed to fluoride [Cooper and Ludwig, 1965].

Cell Differentiation and Functional Morphology

Almost no effects of fluoride on odontogenic cell differentiation were detected at ~50 μ*M* peak plasma fluoride concentrations [Walton and Eisenmann, 1974] and up to 265 μ*M* in culture medium [Bronckers et al., 1984a], although at higher concentrations (3 m*M*) a delay in differentiation was reported [Kerley and Kollar, 1977].

While not strictly an effect on differentiation, effects on ameloblast cytoskeletal components have been reported recently in abstract form [Gibson et al., IADR Meeting, Gothenburg, 2003]. The amelogenin gene is nested within a RhoGAP gene, which regulates intracellular signalling by activation of Rho G protein and elevation of F actin. Fluoride at 4 m*M* for 30 min was shown to inactivate RhoGAP, activating Rho and elevating F actin. In ameloblasts this was localised to actin-rich ameloblast cell junctions and Tomes processes.

While this concentration is relatively high, lesser concentrations could affect the dramatic alterations to functional cell morphology and cell-cell interactions which accompany the transformation from secretory to maturation phases and presumably reflect changes in cell function from secretion to maturation. This may explain changes in the periodicity of ameloblast cell membrane modulation which occurs during fluorosis (10 μ*M* F in plasma) [Denbesten et al., 1985]. The modulation, between smooth and ruffle-ended ameloblasts, is thought to be involved in final crystal growth. This is again especially pertinent since labile fluoride accumulates in enamel precisely at this developmental stage [Weatherell et al., 1975, 1977].

Effect of Fluoride on Matrix Protein Synthesis and Secretion

The effects of fluoride on cell activity, for example, rate of protein secretion, has been examined but with equivocal results [Denbesten, 1986; Aoba et al., 1990; Robinson and Kirkham, 1990; Aoba and Fejerskov, 2002]. However, a direct effect on matrix composition per se is difficult to discern from data published so far. Only amino-acid compositions have been looked at in detail and no substantial changes due to fluoride have been reported. Since these investigations, a number of distinct protein species have emerged as components of the enamel matrix, e.g. amelogenin, enamelin and ameloblastin together with a number of specific degradative enzymes and other proteins such as albumin and αHS2 glycoprotein and small amounts of sulphated proteins [for reviews see Robinson et al., 1998a; Fincham et al., 1999]. The effect of fluoride on the relative concentrations of these species, their alternatively spliced variants and/or their individual functions remains to be investigated in detail.

Interpretation of existing data is also complicated by post-synthetic protein processing including post-translational modification and the controlled degradation prior to maturation, which produces a highly consistent pattern of breakdown products [Robinson et al., 1998a; Fincham et al., 1999]. It is therefore difficult to separate effects on protein production per se from effects on post-synthetic or post-secretory activity.

A particular case in point is the level of matrix phosphorylation. Judging from the similarity of ^{32}P uptake and of two-dimensional protein gel patterns between fluoridated and control enamel organ cultures, fluoride up to 1.325 m*M* in culture medium had little effect on matrix

phosphorylation levels [Denbesten, 1986]. This implies little effect of fluoride on either post-translational phosphorylation or any post-secretory dephosphorylation.

The investigation, however, predated the identification of specific phosphorylated proteins of the matrix, in particular enamelin (2%) [Fukae et al., 1996], which might benefit from further investigation.

Extracellular Matrix Processing and Loss

A major feature of normal enamel development is the almost complete and selective degradation and loss of enamel matrix proteins, particularly the amelogenins. What remains comprises small peptides, amino acids and insoluble tuft protein [for review see Robinson et al., 1998a]. In mature fluorotic enamel this situation was altered with retention of proline-rich components [Eastoe and Fejerskov, 1984; Wright et al., 1989, 1996]. The precise identity of retained molecular species is unknown but, from their amino acid composition, they did not appear to be intact amelogenin and may be a mixture of degradation products [Wright et al., 1989].

Reports concerning developing fluoridated enamel [Drinkard et al., 1983 (370 μM F peak plasma); Denbesten and Crenshaw, 1984; Robinson and Kirkham, 1984b; Denbesten, 1986] revealed a relative increase in 25-kD components in developing enamel containing nascent amelogenin, much of which was mineral-bound [Robinson et al., 2003a]. Smaller components were also retained during maturation, but these data were less clear.

The most likely explanation for these changes is fluoride-induced retention of intact and degraded protein species together with reduced extracellular proteolysis [for review see Robinson and Kirkham, 1990; Aoba and Fejerskov, 2002; Robinson et al., 2003a].

Lowered calcium activity, due to a less soluble mineral phase, has been suggested to slow down proteolysis by Ca^{++}-dependent, secretory stage proteases [Aoba and Fejerskov, 2002]. While this is possible, it is unlikely as a major factor. First, given the relatively high calcium levels and small amount of enzyme present, extremely severe reductions in calcium would be necessary. Second, major protein destruction occurs at transition via a serine protease (kallikrein 4) which is not Ca^{++}-dependent [Simmer and Hu, 2002]. Third, the timescale for protein removal and maturation is hugely variable between species. In the rat incisor this is about 2 weeks, in the cow and pig about 2 months, and in human teeth this may take years. These latter periods would appear to be quite sufficient for complete processing and removal of matrix in the order of only hundreds of micrograms [Robinson and Kirkham, 1984a, 1990]. It should also be noted that porcine enamel did not appear to mineralise to the same extent as other species, attaining only 55% mineral by weight as opposed to 80–90%. This should be borne in mind when using the pig as a model for fluorosis [Kirkham et al., 1988; Robinson and Kirkham, 1990].

Since direct inhibition of enzyme activity has not been demonstrated convincingly [Drinkard et al., 1983; Gerlach et al., 2000], it is likely that enhanced protein interaction with the mineral, described below, is responsible for both protein retention and reduced proteolysis in fluorosed tissue. Increased binding of both undegraded amelogenin [Robinson et al., 2003a] and enamel proteases to the mineral phase [Brookes et al., 1998; Aoba and Fejerskov, 2002] have been reported. This may be especially important at the transition/maturation stage, where final degradation occurs via a specific serine protease (kallikrein 4) and where fluoride accumulates selectively.

Effect of Retained Protein

The result of fluoride-induced protein retention may also explain the incomplete crystal growth which characterises fluorosis, since it has been demonstrated that matrix removal is a necessary prerequisite for unimpaired crystal growth in enamel [Robinson et al., 1989] and synthetic apatites [Aoba et al., 1987]. In this context, one area worthy of further exploration is the role of ameloblastin. Fluorosis involves incomplete crystal growth at prism peripheries and it is at this site that degradation products of ameloblastin accumulate during development [Uchida et al., 1997; Robinson et al., 1998b]. Impaired removal of ameloblastin due to fluoride could be responsible for incomplete crystal growth in this region.

Effect of Fluoride on the Mineral Phase

Initiation of Precipitation during Secretion

While it is established that mature enamel crystals comprise a substituted calcium hydroxyapatite, the precise nature of initial mineral phases, whether in enamel or dentine, is still a matter of some controversy. These range from amorphous short range order calcium phosphates [Posner, 1985] through brushite-like phases to octacalcium phosphate [Brown et al., 1987; Iijima et al., 1992; Johnsson and Nancollas, 1992]. These are often said to be stabilised by carbonate or magnesium. Whatever the nature of this phase, there seems to be general agreement that the presence of fluoride ion during initial deposition can delay formation of an initial apatite precursor, proba-

bly by stabilising very early precursor entities [Bachra and Fischer, 1969]. Work by Bronckers et al. [1984b] using hamster tooth germs is consistent with this view and showed that protein matrix formed under an elevated fluoride regime did not mineralise at all unless fluoride was removed, indicating a reversible effect on the matrix-associated initiation process.

Precisely how and where initiation occurs is still a matter of discussion. Recently, Robinson et al. [2003c] suggested that crystal formation in enamel may involve fusion of precursor protein/mineral-ion subunits, the established degradative processing of the matrix facilitating initial mineral precipitation. Stabilisation of these protein mineral subunits by fluoride per se would also prevent or delay initiation.

Crystal Growth

Once the initial mineral phase has formed, fluoride facilitates more rapid deposition [Bachra and Fischer, 1969; Varughese and Moreno, 1981]. This may be due to fluoride-induced conversion of acidic precursors such as amorphous calcium phosphate or octacalcium phosphate to apatite [Iijima et al., 1992]. Perhaps a more likely explanation would be the higher relative supersaturation of tissue fluids with respect to a precipitating fluoridated mineral phase. This would be facilitated by the effect of fluoride in reducing the incorporation of destabilising extraneous ions such as carbonate [Nikiforuk and Grainger, 1965].

Stimulation of crystal growth during early secretion is supported by the work of Bronckers et al. [1984b], who showed, in culture, that with access to fluoride (up to 26.5 μM), partially mineralised matrix became hypermineralised. This would be consistent with a more rapid mineral deposition due to a higher relative supersaturation for fluoride-containing mineral. While much in vitro data suggests that fluoride can increase apatite crystal growth in the a and b axes [Eanes and Hailer, 1998], there is little evidence that, at least in enamel, this results in significant alteration to apatite crystal morphology or size [Yanagisawa et al., 1989]. Such changes which have been reported were restricted to the outer enamel and attributed to post-eruptive alterations [Yanagisawa et al., 1989].

Effect of Fluoride on Mineral Properties

During mineral deposition, fluoride is incorporated into the growing hydroxyapatite crystals either by accretion or by heteroionic substitution.

Fluoride is known to occupy the hydroxyl site in the long c axis of the crystal. The charge symmetry and high negative charge density allow a better fit in the lattice compared with the larger asymmetric hydroxyl ion. The effects are profound. The sense of the hydroxyl columns is altered such that adjacent hydroxyls will hydrogen bond to the fluoride ion. In addition, protons associated with acid phosphate groups might be more tightly orientated towards the fluoride ion [Posner et al., 1963; Kay et al., 1964; Van der Lugt et al., 1971].

In terms of overall crystal behaviour, energy levels are much reduced. This explains the lower solubility product for fluoridated compared with non-fluoridated mineral and the fact that the crystal is less reactive. With regard to resorption in dentine and dissolution in caries, the fluoridated crystal is much more acid-resistant. In addition, the larger asymmetric substituent, carbonate tends to be excluded from the crystal, further increasing stability [McCann and Bullock, 1957].

Magnesium might be expected to exert a similar effect to carbonate since it does not fit well in the lattice (about 0.2% maximum). However, an increase in fluoride is usually accompanied by an increase in magnesium content [McCann and Bullock, 1957; Robinson et al., 1983]. This may relate to the fact that magnesium is at highest concentrations during secretion [Hiller et al., 1975] and like fluoride shows some selective uptake during transition [Robinson et al., 1984; Kirkham et al., 1988]. This has been attributed to close affinity of magnesium for fluoride during incorporation into the crystals [Okazaki, 1987]. It may also be surface-located [Neuman and Mulryan, 1971], its higher concentrations being due to specific surface complexes and/or by a greater surface area of the rougher crystal surfaces [Kirkham et al., 2001] (see below).

Matrix-Mineral Interactions

During enamel maturation, crystal growth, especially in the final stages, is clearly compromised since fluorosis is characterised by greater intercrystalline space, particularly at the prism peripheries [Fejerskov et al., 1977]. Since enamel matrix removal appears to be a prerequisite for normal crystal growth [Aoba et al., 1987; Robinson et al., 1989], impairment of crystal development in vivo has been associated with the demonstrated retention of mineral-bound protein matrix [Drinkard et al., 1983; Denbesten, 1986; Robinson et al., 2003a]. The mechanism of enhanced retention and the molecular species involved (see above) are not yet clear. Fluoridated mineral may bind proteins more effectively [Tanabe et al., 1988] due to

greater hydrogen bonding or a less polar surface [Wu and Nancollas, 1999]. Increased magnesium, if located in the Helmholz double layer, could provide cationic bridging for proteins. This does not exclude the possibility that increased magnesium per se could impair growth [Bachra and Fischer, 1969].

More recent work using the atomic force microscope (AFM) has suggested that fluoride could also influence crystal surface morphology and perhaps the mode of crystal growth. This might contribute towards a unifying view of fluoride action. AFM studies of crystal surfaces at the molecular level have indicated that during enamel development, in the rat, the surface roughness of crystals normally decreased in moving from secretion to maturation phases [Kirkham et al., 1998]. This may have resulted from changes in matrix binding but may also reflect a decrease in kink and step site density due to a growth/healing process perhaps involving a shift from polynuclear towards spiral growth.

Enamel produced under fluorotic conditions, however, did not show such a reduction in roughness. Not only was roughness greater than in non-fluorotic teeth but it was also maintained throughout development [Kirkham et al., 2001]. Since fluoride is taken up selectively during transition and maturation stages [Weatherell et al., 1977], local supersaturation levels would be relatively high in terms of the fluoridated depositing phase. This high supersaturation would favour polynuclear growth and thus increased surface roughening.

Such increased roughening could, together with changes in crystal surface chemistry, account for the increased magnesium typical of fluorosed enamel, a view supported by the fact that magnesium, like fluoride, is selectively taken up at the transition/maturation stage [Hiller et al., 1975; Robinson et al., 1984; Kirkham et al., 1988]. The increased surface area due to roughening could also facilitate protein binding/retention [Gathercole et al., 1996].

Use of the AFM in chemical force mode has also revealed novel information concerning enamel crystal surface properties. Using carboxyl- or hydroxyl-functionalised tips their binding strength to apatite surfaces was measured as a function of pH. This revealed pK values for apatite surfaces an order of magnitude lower than solution phosphate, implying greater electronegativity. When fluoride was present, binding values were higher and pK values were even lower, indicating further increased, presumably hydrogen, bonding with phosphate groups or fluoride itself [Robinson et al., 2003b] somewhat similar shifts to those seen in bulk synthetic systems [Wu et al.,1991]. Such studies offer important possibilities for future studies of fluoride-mediated changes to crystal surface properties, not only of crystals, but of the modulating organic matrix.

While much of the consideration of the effect of fluoride resides with its effect on the lattice proper, the roughness findings described above also suggest that the crystal surface/fluid interface should be considered. Given the great propensity for fluoride to form hydrogen bonds it is likely that it could affect ligand binding and exchange with the Helmholz/Gouy-Chapman layers and thus with the lattice itself.

Dentine

The effect of fluoride on dentine is only detectable at concentrations much higher than those required for enamel fluorosis. Overall effects, however, are similar in that hypomineralisation results [Fejerskov et al., 1979].

Matrix Synthesis and Composition

While no fluoride-mediated alterations to the main extracellular component, type 1 collagen, have been reported, specific changes to non-collagenous components do occur. Perhaps of greatest current interest is a reported effect on the dentine phosphoproteins [Milan et al., 1999]. Rats rendered fluorotic by dietary fluoride revealed lower molecular sizes for dentine phosphoprotein (phosphophoryn) which, together with lowered phosphate content, was attributed to a lower degree of phosphorylation. Investigations into casein kinase II and alkaline phosphatase – both enzyme types present in developing dentine – also revealed fluoride-mediated inhibition [Milan et al., 2001]. Clearly fluoride is capable of affecting the metabolism of dentine phosphoproteins. The reduction of phosphorylation, in particular, might well decrease mineral ion binding and probably their capacity for crystal initiation.

Analysis of proteoglycans from fluorosed rat dentine in vivo revealed no alterations to the protein core. However, glycosylaminoglycans (GAGs) appeared to be smaller and more anionic, possibly due to the additional presence of dermatan and heparan sulphate [Hall et al., 1996]. Interaction of these GAGs with mineralising collagen, the main extracellular matrix component, may be affected, possibly restricting mineral initiation, while binding to the mineral phase could result in less mineral deposition.

Mineral Phase

The mineral phase of dentine takes the form of very small apatite crystals 50 × 70 × 5 nm, embedded within a highly cross-linked type I collagen matrix. There is little direct information on fluorosed or fluorotic dentine mineral. Early reports did, however, suggest that it might contain reduced levels of carbonate and elevated magnesium similar to fluorotic enamel [McCann and Bullock, 1957]. The implications would be related as in enamel to a reduction in the supersaturation levels required for mineral precipitation and crystal growth with a concomitant reduction in acid solubility.

Concluding Remarks

Despite a very large and often confusing literature concerning the mechanisms which lead to dental fluorosis a relatively clear and well-supported concept is emerging, at least for exposure to concentrations which are not overtly toxic.

It seems likely that, at least in enamel, the effect on the developing mineral phase per se coupled with associated effects on the surrounding and modulating protein matrix could account for most of the observed effects of the fluoride ion on tooth development.

The most obvious feature of fluorosis – impaired growth of apatite crystals – seems attributable to retention of modulating matrix proteins through enhanced binding of mineral to matrix proteins and/or enhanced binding of the proteases responsible for processing prior to matrix removal.

There are still many unanswered questions, however. Information with regard to effects on specific molecular species is still sparse and more detailed studies of the effect of fluoride on the recent unsuspected substructure of apatite crystal surfaces is unclear. With regard to the effect of fluoride on odontogenic cells, the latest information suggests that functional cell structure might be altered at a stage affecting final crystal growth and matrix withdrawal. The following lists include those areas where information is still lacking or unclear.

Avenues for Future Research

Effect of Fluoride on:

- Effect of fluoride on cytoskeletal components through the RhoGAP system.
- MAPK kinase phosphatase and similar mitosis-sustaining pathways in odontogenesis cells.
- Relative secretion/amounts of specific protein species with established sequences.
- Alternative splicing of matrix proteins.
- Post-translational processing of specific proteins (phosphorylation, glycosylation, sulphation).
- Post-secretory processing of specific proteins (proteolysis, dephosphorylation, de-glycosylation).
- Surface/molecular morphology of crystals.
- Surface chemistry of crystals (double layer and water structure).
- Specific protein-mineral interactions (motif changes?).
- Regrowth of fluorosed crystals (re-activation of crystal surfaces).
- Effect of increased magnesium on crystal growth.

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8. REGULATIONS AND ADVISORIES

No international regulations pertaining to fluorides were found. The national and state regulations and guidelines regarding fluorides, hydrogen fluoride, and fluorine in air, water, and other media are summarized in Table 8-1.

A chronic-duration oral MRL of 0.05 mg fluoride/kg/day has been derived for fluoride. This MRL is based on a NOAEL of 0.15 mg fluoride/kg/day and a LOAEL of 0.25 mg fluoride/kg/day for skeletal effects (increased fracture rate) (Li et al. 2001). The MRL was derived by dividing the NOAEL by an uncertainty factor of 3 to account for human variability.

An acute-duration inhalation MRL of 0.02 ppm fluoride has been derived for hydrogen fluoride. This MRL is based on a minimal LOAEL of 0.5 ppm for upper respiratory tract inflammation in humans exposed to hydrogen fluoride for 1 hour (Lund et al. 1997, 1999). The MRL was derived by dividing the unadjusted LOAEL by an uncertainty factor of 30 (3 for a use of a minimal LOAEL and 10 to account for human variability).

An acute-duration inhalation MRL of 0.01 ppm has been derived for fluorine. This MRL is based on a NOAEL of 10 ppm for respiratory irritation in humans exposed to fluorine for 15 minutes (Keplinger and Suissa 1968). The MRL was derived by dividing the 24-hour adjusted NOAEL of 0.1 ppm by an uncertainty factor of 10 to account for human variability.

EPA (IRIS 2003) derived an oral reference dose (RfD) of 0.06 mg/kg/day for fluorine (soluble fluoride). The RfD was based on a NOAEL of 0.06 mg/kg/day and a LOAEL of 0.12 mg/kg/day for the cosmetic effect of dental fluorosis in children (Hodge 1950). The NOAEL was divided by an uncertainty factor of 1 to derive the RfD.

Table 8-1. Regulations and Guidelines Applicable to Fluoride, Sodium Fluoride, Hydrogen Fluoride, and Fluorine

Agency	Description	Information	Reference
INTERNATIONAL			
Guidelines:			
IARC	Carcinogenicity classification		IARC 1987
	Fluoride and sodium fluoride	Group 3[a]	
WHO	Drinking water guideline		WHO 2001
	Fluoride	1.5 mg/L	
NATIONAL			
Regulations and Guidelines:			
a. Air			
ACGIH	TLV-TWA		ACGIH 2000
	Fluoride	2.5 mg/m^3	
	Fluorine	1.0 ppm	
	STEL (ceiling)		
	Fluorine	2.0 ppm	
	Hydrogen fluoride	3.0 ppm	
EPA	Accidental release prevention		EPA 2001b
	Threshold quantity		40CFR68.130
	Fluorine	1,000 pounds	Table 1
	Hydrogen fluoride	1,000 pounds	
	Accidental release prevention		EPA 2001a
	Toxic end point		40CFR68
	Fluorine	0.0039 mg/L	Appendix A
	Hydrogen fluoride	0.0160 mg/L	
OSHA	PEL (8-hour TWA)		OSHA 2001c
	General industry		29CFR1910.1000
	Fluoride	2.5 mg/m^3	Table Z-1
	Fluorine	0.2 mg/m^3	
	Hydrogen fluoride	2.0 mg/m^3	
	PEL (8-hour TWA)		OSHA 2001f
	Construction industry		29CFR1926.55
	Fluoride	2.5 mg/m^3	Appendix A
	Fluorine	0.2 mg/m^3	
	Hydrogen fluoride	2.0 mg/m^3	
	PEL (8-hour TWA)		OSHA 2001a
	Shipyards		29CFR1915.1000
	Fluoride	2.5 mg/m^3	Table Z
	Fluorine	0.2 mg/m^3	
	Hydrogen fluoride	2.0 mg/m^3	
	Highly hazardous chemicals		OSHA 2001d
	Threshold quantity		29CFR1910.119
	Fluorine	1,000 pounds	Appendix A
	Highly hazardous chemicals		OSHA 2001e
	Threshold quantity		29CFR1926.64
	Hydrogen fluoride	1,000 pounds	Appendix A

Table 8-1. Regulations and Guidelines Applicable to Fluoride, Sodium Fluoride, Hydrogen Fluoride, and Fluorine

Agency	Description	Information	Reference
NATIONAL *(cont.)*			
OSHA	Brazing and gas welding fluxes shall have a cautionary wording to indicate that they contain fluorine compounds		OSHA 2001b 29CFR1910.252(c)(1)
NIOSH	REL (TWA)		NIOSH 2001a
	Fluorine	0.2 mg/m^3	NIOSH 2001b
	Hydrogen fluoride	2.5 mg/m^3	NIOSH 2001c
	Sodium fluoride	2.5 mg/m^3	
	IDLH		NIOSH 2001a
	Fluorine	25 ppm	NIOSH 2001b
	Hydrogen fluoride	30 ppm	NIOSH 2001c
	Sodium fluoride	250 ppm	
USC	HAP		USC 2001 42USC7412
b. Water			
EPA	BPT effluent limitation—fluoride		EPA 2001c
	Maximum for 1 day	6.1 kg/kkg	40CFR415.82
	Average of daily values for 30 consecutive days	2.9 kg/kkg	
	Effluent limitation—fluoride		EPA 2001e
	Maximum for 1 day	75 mg/L	40CFR422.42
	Average of daily values for 30 consecutive days	25 mg/L	
	Groundwater protection standards at inactive uranium processing sites—listed constituents include fluorine and hydrogen fluoride		EPA 2001f 40CFR192 Appendix I
	MCLG—fluoride	4.0 mg/L	EPA 2001j 40CFR141.51(b)
	MCL—fluoride	4.0 mg/L	EPA 2001k 40CFR141.62(b)
	Secondary MCL—fluoride	2.0 mg/L	EPA 2001l 40CFR143.3
	Water pollution—hazardous substance designation	Hydrogen fluoride Sodium fluoride	EPA 2001r 40CFR116.4
c. Food			
EPA	Pesticides—fluorine compounds; residue tolerances		EPA 2001n 40CFR180.145
	Apricots, beets, blackberries, blueberries, boysenberries, broccoli, brussels sprouts, cabbage, cauliflower, citrus fruits, collards, cranberries	7 ppm	

Table 8-1. Regulations and Guidelines Applicable to Fluoride, Sodium Fluoride, Hydrogen Fluoride, and Fluorine

Agency	Description	Information	Reference
NATIONAL *(cont.)*			
EPA	Pesticides—fluorine compounds; residue tolerances		EPA 2001n 40CFR180.145
	Cucumbers, dewberries, eggplant, grapes, kale, kohlrabi, lettuce, loganberries, melons, nectarines, peaches, peppers, plums, pumpkins, radish, raspberries, rutabaga, squash, strawberries, tomatoes, turnip, youngberries	7 ppm	
	Potatoes	2 ppm	
	Potatoes, processing waste	22 ppm	
	Kiwifruit	15 ppm	
FDA	Adhesive component, indirect food additive—for use only as bonding agent for aluminum foil, stabilizer, or preservative	Total fluoride from all sources not to exceed 1% by weight of the finished adhesive	FDA 2000e 21CFR175.105(c)(5)
	Hydrogen fluoride		
	Sodium fluoride		
	Bottled water—no fluoride added	Temperature[b] / mg/L 53.7–below / 2.4 53.8–58.3 / 2.2 58.4–63.8 / 2.0 63.9–70.6 / 1.8 70.7–79.2 / 1.6 79.3–90.5 / 1.4	FDA 2000g 21CFR165.110
	Bottled water—fluoride added	Temperature[b] / mg/L 53.7–below / 1.7 53.8–58.3 / 1.5 58.4–63.8 / 1.3 63.9–70.6 / 1.2 70.7–79.2 / 1.0 79.3–90.5 / 0.8	
	Over-the-counter drug products Labeling—fluoride, fluorine, and sodium fluoride		FDA 2000b 21CFR355.50 FDA 2000c 21CFR355.60
	Over-the-counter drug products Testing—fluoride		FDA 2000d 21CFR355.70
	Over-the-counter drug products Active ingredient—fluorine, hydrogen fluoride, and sodium fluoride		FDA 2000a 21CFR355.10 FDA 2000f 21CFR310.545(a)(2)

Table 8-1. Regulations and Guidelines Applicable to Fluoride, Sodium Fluoride, Hydrogen Fluoride, and Fluorine

Agency	Description	Information	Reference
NATIONAL *(cont.)*			
FDA	Surface component, food contact—sodium fluoride for use as preservative only		FDA 2000h 21CFR177.2800 (d)(5)
d. Other			
ACGIH	Carcinogenicity classification		ACGIH 2000
	Fluoride	A4[c]	
	BEI		
	Fluorides in urine		
	Prior to shift	3 mg/g creatinine	
	End of shift	10 mg/g creatinine	
CPSC	Requirements for child-resistant packaging for household products containing elemental fluoride	More than 50 mg and more than 0.5%	CPSC 2001 16CFR1700
DOT	Hazardous materials		DOT 2001 40CFR172.101
	Reportable quantity		Appendix A
	Fluorine	10 pounds	
	Hydrogen fluoride	100 pounds	
	Sodium fluoride	1,000 pounds	
EPA	RfD—fluorine	$6x10^{-2}$ mg/kg/day	IRIS 2003
	Toxic chemical release reporting; Community Right-to-Know—effective date		EPA 2001q 40CFR372.65
	Fluorine	01/01/95	
	Hydrogen fluoride	01/01/87	
	Contaminated soil—fluoride	Concentrations greater than 10 times UTS	EPA 2001d 40CFR268.49(f)
	Hazardous waste—health based limits for exclusion of waste-derived-residue		EPA 2001g 40CFR266 Appendix VII
	Fluorine residue concentration limit	4.0 mg/kg	
	Hazardous waste—identification and listing		EPA 2001h 40CFR261.33(e)
	Fluorine	P056	EPA 2001i
	Hydrogen fluoride	U134	40CFR261.33(f)
	Pesticides—residue tolerances	Not more than 25% of	EPA 2001m
	Sodium fluoride	pesticide formulation	40CFR180.1001(d)
	Superfund—reportable quantity		EPA 2001o
	Fluorine	1 pound	40CFR302.4
	Hydrogen fluoride	5,000 pounds	Appendix A
	Sodium fluoride	5,000 pounds	

Table 8-1. Regulations and Guidelines Applicable to Fluoride, Sodium Fluoride, Hydrogen Fluoride, and Fluorine

Agency	Description	Information	Reference
NATIONAL *(cont.)*			
EPA	Superfund—extremely hazardous		EPA 2001p
	Reportable quantity		40CFR355
	Fluorine	10 pounds	Appendix A
	Hydrogen fluoride	100 pounds	
	Threshold planning quantity		
	Fluorine	500 pounds	
	Hydrogen fluoride	100 pounds	
STATE			
a. Air			
Connecticut	HAP—fluoride, fluorine, and hydrogen fluoride		BNA 2001
Hawaii	Air contaminant—hydrogen fluoride		BNA 2001
Idaho	Toxic air pollutants		BNA 2001
	Fluoride		
	OEL	2.5 mg/m^3	
	EL	0.167 pounds/hour	
	AAC	0.125 mg/m^3	
	Fluorine		
	OEL	2.0 mg/m^3	
	EL	0.133 pounds/hour	
	AAC	0.1 mg/m^3	
Michigan	PEL (TWA)		BNA 2001
	Fluoride	2.5 mg/m^3	
	Fluorine	0.2 mg/m^3	
	Hydrogen fluoride	3.0 ppm	
Montana	Air contaminant (TWA)		BNA 2001
	Fluoride	2.5 mg/m^3	
	Fluorine	0.2 mg/m^3	
	Hydrogen fluoride	2.0 mg/m^3	
New Mexico	Toxic air pollutant		BNA 2001
	Fluorides		
	OEL	2.5 mg/m^3	
	Emissions	0.167 pounds/hour	
	Fluorine		
	OEL	2.0 mg/m^3	
	Emissions	0.133 pounds/hour	
New York	Air contaminant (TLV)		BNA 2001
	Fluoride	2.5 mg/m^3	
	Fluorine	0.2 mg/m^3	
	Hydrogen fluoride	2.0 mg/m^3	

Table 8-1. Regulations and Guidelines Applicable to Fluoride, Sodium Fluoride, Hydrogen Fluoride, and Fluorine

Agency	Description	Information		Reference
STATE *(cont.)*				
Washington	Toxic air pollutant—ASIL			BNA 2001
	Fluoride	8.3 $\mu g/m^3$		
	Fluorine	5.3 $\mu g/m^3$		
	Hydrogen fluoride	8.7 $\mu g/m^3$		
	PEL			BNA 2001
	Fluoride	2.5 mg/m^3		
	Fluorine	0.2 mg/m^3		
	Hydrogen fluoride (STEL)	3.0 ppm		
Wisconsin	Emission rate (pounds/hour)	<25 feet	>25 feet	BNA 2001
	Fluoride	0.2088	0.8640	
	Fluorine	0.1656	0.6720	
	Hydrogen fluoride	0.1272	0.4800	
b. Water				
Alaska	MCL—fluoride	4.0 mg/L		BNA 2001
	Secondary MCL—fluoride	2.0 mg/L		
Arizona	Drinking water guideline—fluoride	4.0 mg/L		HSDB 2003
	Reporting limit—fluoride	2.0 mg/L		BNA 2001
California	Drinking water standards—fluoride	2.0 mg/L		HSDB 2003
Connecticut	MCL—fluoride	4.0 mg/L		BNA 2001
Delaware	Drinking water standards—fluoride	1.8 mg/L		HSDB 2003
Georgia	MCL—fluoride	4.0 mg/L		BNA 2001
Hawaii	Drinking water standards—fluoride	1.4–2.4 mg/L		HSDB 2003
Idaho	Groundwater quality standards—fluoride	4.0 mg/L		BNA 2001
Kansas	Agriculture—fluoride			BNA 2001
	Livestock	2.0 mg/L		
	Irrigation	1.0 mg/L		
	Public health food—fluoride			
	Domestic water supply	2.0 mg/L		
Maine	Drinking water guideline—fluoride	2.4 mg/L		HSDB 2003
	Maximum exposure guideline	2.4 mg/L		BNA 2001
	Action level	1.2 mg/L		
Mississippi	Groundwater standards—fluoride	4.0 ppm		BNA 2001
Nebraska	MCL—fluoride	4.0 mg/L		BNA 2001

Table 8-1. Regulations and Guidelines Applicable to Fluoride, Sodium Fluoride, Hydrogen Fluoride, and Fluorine

Agency	Description	Information	Reference
STATE *(cont.)*			
New Jersey	Groundwater quality criteria—fluoride	2.0 mg/L	BNA 2001
	PQL—fluoride	0.5 mg/L	
New York	Groundwater effluent limitations—fluoride	3.0 mg/L	BNA 2001
	MCL—fluoride	2.2 mg/L	BNA 2001
North Carolina	Drinking water standards—fluoride	4.0 mg/L	HSDB 2003
North Dakota	MCL—fluoride	4.0 mg/L	BNA 2001
Oklahoma	MCL—fluoride	4.0 mg/L	BNA 2001
Pennsylvania	Drinking water standards—fluoride	2.0 mg/L	HSDB 2003
Rhode Island	MCLG—fluoride	4.0 ppm	BNA 2001
	MCL—fluoride	4.0 ppm	
South Dakota	Groundwater quality standards—fluoride	2.4 mg/L	BNA 2001
Tennessee	MCL—fluoride	4.0 ppm	BNA 2001
Texas	MCL—fluoride	4.0 mg/L	BNA 2001
Utah	Groundwater standards	4.0 mg/L	BNA 2001
	MCL—fluoride	4.0 mg/L	BNA 2001
Vermont	Groundwater quality standards—fluoride		BNA 2001
	Enforcement standard	4.0 mg/L	
	Preventive action level	2.0 mg/L	
	MCL—fluoride	4.0 mg/L	BNA 2001
Washington	MCL—fluoride	4.0 mg/L	BNA 2001
West Virginia	Groundwater standards	Not to exceed 4.0 mg/L	BNA 2001
Wisconsin	MCLG—fluoride	4.0 mg/L	BNA 2001
	MCL—fluoride	4.0 mg/L	
	Groundwater standards—fluoride		BNA 2001
	Enforcement standard	4.0 mg/L	
	Preventive action limit	0.8 mg/L	
c. Food		No data	
d. Other			
Connecticut	Use of pesticides; control of registrations and uses—sodium fluoride	For use as a wood preservative	BNA 2001

Table 8-1. Regulations and Guidelines Applicable to Fluoride, Sodium Fluoride, Hydrogen Fluoride, and Fluorine

Agency	Description	Information	Reference
STATE *(cont.)*			
Minnesota	Hazardous substance—fluoride (as F, as dust), fluorides (inorganic), fluorine, and hydrogen fluorine		BNA 2001
New Jersey	Hazardous substance—fluorine and hydrogen fluoride		BNA 2001

[a]Group 3: not classifiable as to its carcinogenicity to humans
[b]Temperature: annual average of maximum daily air temperatures (•F)
[c]A4: not classifiable as a human carcinogen

AAC = acceptable ambient concentrations; ACGIH = American Conference of Governmental Industrial Hygienists; ASIL = acceptable source impact levels; BEI = biological exposure indices; BNA = Bureau of National Affairs; BPT = best practicable control technology; CFR = Code of Federal Regulations; CPSC = Consumer Product Safety Commission; DOT = Department of Transportation; EL = emissions levels; EPA = Environmental Protection Agency; FDA = Food and Drug Administration; HAP = hazardous air pollutant; HSDB = Hazardous Substances Data Bank; IARC = International Agency for Research on Cancer; IDLH = immediately dangerous to life and health; IRIS = Integrated Risk Information System; MCL = maximum contaminant level; MCLG = maximum contaminant level goal; NIOSH = National Institute for Occupational Safety and Health; OEL = occupational exposure limit; OSHA = Occupational Safety and Health Administration; PEL = permissible exposure limit; PQL = practical quantitation level; REL = recommended exposure limit; RfD = reference dose; STEL = short term exposure limit; TLV = threshold limit values; TWA = time-weighted average; USC = United States Code; UTS = universal treatment standards; WHO = World Health Organization

Fluoride content of powdered infant formula meets Australian Food Safety Standards

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Abstract

Objectives: To identify the fluoride content of powdered formula for infants 0-12 months in products available from Brisbane stores in 2006/07 and compare this with the fluoride content of infant formula products available in Australia 10 years earlier.

Methods: A range of available infant formula powders were collected from major supermarkets and chemists in Brisbane, Queensland. The fluoride levels in infant formula powder samples were determined using a modification of the micro-diffusion method of Silva and Reynolds[1] utilising perchloric acid and silver sulphate and measured with an ion selective (fluoride) electrode/meter. Fluoride content both prior to and after reconstitution, as well as estimated daily intake according to age was calculated.

Results: Formula samples contained an average of 0.49 µg F/g of powder (range 0.24–0.92 µg F/g). After reconstitution with water containing 0mg/L fluoride, the fluoride content averaged 7.09µg F/100mL (range 3.367–22.72 µg F/100mL). Estimated infant fluoride intakes ranged from 0.0039 mg/kg/day for a 6-12 month old infant when reconstituting milk-based formula with non-fluoridated water (0 mg/L), to 0.1735 mg/kg/day for a 0-3 month old infant when reconstituting soy-based formula with fluoridated water (1.0 mg/L).

Conclusions: Infant formula powders contain lower levels of fluoride than previously found in Australia in 1996.

Implications: This confirms that infants consume only a small amount of fluoride from milk-based powdered infant formula. Although soy-based infant formulas contain more fluoride than milk-based products, the levels still comply with national food standards.

Key words: Fluoride, infant formula, oral health, water fluoridation, fluorosis, dental, micro-diffusion, ion selective electrode.

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There are numerous guideline values for the level of exposure to fluoride that maximises health benefits (tooth decay prevention) and minimises adverse effects (dental fluorosis).[2,3,4] In Australia, 64% of children experience tooth decay (dental caries) that causes concerns from mild discomfort through to debilitating pain as well as reduced capacity to chew, talk and smile. In comparison, approximately 22% of children experience some form of dental fluorosis.[9,17] Dental fluorosis is mottling of teeth that may result from excessive fluoride being ingested while teeth are developing and may be of aesthetic concern. The NHMRC dietary guidelines for Australia and New Zealand, recommend fluoride adequate intake (AI) for infants which ranges from 0.01 mg/day (birth to six months) to 0.5 mg/day (seven to 12 months). The upper limit (UL) is 0.7 mg/day and 0.9 mg/day, respectively.[3] Other authors have also reported guidance values 0.05–0.07 mg F/kg body weight/day and ULs of up 0.10 mg F/kg body weight/day.[1,4,5]

In recent years it has become widely accepted that little pre-eruptive benefit to tooth enamel is achieved through the ingestion of fluoride, as the majority of benefit occurs from frequent exposure of the tooth enamel to fluorides post eruption.[6] Therefore, benefit from fluoride will not begin until the first teeth erupt, at about six months of age at which time fluoride intake may come from an increasing variety of sources with the introduction of solid foods. Risk from excess fluoride ingestion in the first 12 months has been shown to have an association with deciduous teeth but not with permanent teeth as the critical period in which permanent teeth are most vulnerable does not begin until 15 to 21 months of age.[7,8] This is supported by recent studies that show that reconstitution of infant formula with fluoridated water at 1 mg/L has little impact on fluorosis levels of permanent teeth, compared to other sources of fluoride ingestion, such as toothpaste ingestion and fluoride supplement use.[9,10] Despite lack of agreement on guideline values and varying units of measurement, fluoride intake from infant formula has been frequently identified as a potential source of excess fluoride for infants depending on production processes and fluoride concentration of water used for reconstitution.[4,7,11]

In 1996, Silva and Reynolds investigated fluoride concentrations in infant formula in Australia and found that, when water fluoridated at 1 mg/L was used to reconstitute

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their formula samples, all exceeded their suggested UL of fluoride intake. In 1999, the NHMRC made a recommendation to develop a strategy to reduce excessive intake of fluoride from infant formula in Australia.[6] The Australia New Zealand Food Standards Code was amended to include mandatory dental fluorosis risk labelling of powdered or concentrated infant formula if it contains more than 17μg F/100 kilojoules (kJ) prior to reconstitution.[12] Since that time there has been no reported investigation of the levels of fluoride in powdered or concentrated infant formula products commercially available in Australia. This study sought to redress this deficit.

Methods

One tin of every infant formula that was present on the shelves of two different major supermarkets in Brisbane was purchased in March 2006. Where possible, a different batch number was obtained in another two stores of the same supermarket chain to achieve a sample of 33 different infant formula products. Two chemist chain stores were also accessed to identify other common brands and different batches of products (June 2007). This produced a total sample of 53 tins of infant formula powder (15 products were double sampled, one triple sampled and one had four samples). Product labels identified country of manufacture as New Zealand, Ireland/Singapore, France/Germany/Netherlands and Australia.

Fluoride in the infant formula powders was determined using a modification of the micro-diffusion method of Silva and Reynolds utilising perchloric acid and silver sulphate. Square polystyrene Petri dishes with unvented lids to prevent loss of liberated hydrofluoric acid (100 mm x 100 mm x 18 mm) were used throughout this work and were pre-cleaned by soaking in 5% perchloric acid overnight, rinsed with deionised water and oven dried prior to use.

Analytical Reagent (AR) grade perchloric acid and silver sulphate may contain low levels of fluoride as an impurity and must be processed to reduce the fluoride contamination to acceptable levels. This was achieved by 0.3 g finely ground silver sulphate and 14 mL of 48% perchloric acid (prepared by diluting concentrated 70% perchloric acid) was placed in a Petri dish and mixed with a stirring rod. The lid was placed slightly ajar on top of the Petri dish (to allow contaminant HF vapours to escape) and placed in an air oven at 50°C overnight. After the heating process in the air oven overnight, the perchloric acid and silver sulphate was ready to use.

At least 12 x 12 drops of 0.5M sodium hydroxide solution were pipetted to the inside of the Petri dish lids using an eight channel Finn-pipette, ensuring that the 144 drops were evenly spaced. The Finn-pipette was set so the volume of each drop was approximately 3.5 μL resulting in a final volume of 0.5M sodium hydroxide spotted onto the lid of approximately 0.5 mL. The lids were placed, with drops facing upwards, into a clean air oven for at least two hours to allow the moisture in the sodium hydroxide solution to evaporate.

A weight of 0.35 g of infant formula powder was transferred to the prepared (decontaminated) perchloric acid/silver sulphate mixture and covered with the spotted lid as quickly as possible. The mixture was evenly dispersed by swirling and then placed in a clean air oven at 50°C for 20hrs. At the completion of the heating process, 1.5 mL deionised water and 1.5 mL TISAB reagent (total ionic strength adjustment buffer) was added to the Petri lid and manually swirled gently until all the crystals were dissolved (about five minutes per sample) as the solution does not entirely cover the bottom of the Petri lid. The 3 mL extract on the lid was then transferred into an acid washed 15 mL HDPE plastic tube and the screw cap was fixed to avoid contamination. The fluoride content in the 3 mL extract was then measured directly from the 15 mL HDPE plastic tube using the ISE electrode/meter. Three certified reference materials (CRM) were used during this study and provided the following results for fluoride; NCS DC 73350 (powdered poplar leaves): requires 22±4 mg/kg, found 20±0.5 mg/kg; Lypocheck Urine (Ref 405, Lot69112): requires 7.7±0.4 mg/L, found 7.7±0.25 mg/L; Seronorm Urine (Ref 201205, Lot 0511545): requires 4.0 mg/L, found 3.7±0.07 mg/L.

Queensland Health formula feeding guidelines within the Personal Health Record[13] were used to calculate the average consumption of reconstituted formula for 0-2 months (150 mL/5.1 kg/day = 765 mL/day), 3-6 months (120 mL/7kg/day = 840 mL/day) and 7-12 months (110 mL/809 kg/day = 979 mL/day).

Table 1: Fluoride content of milk-based (MB) and soy-based (SB) infant formula when reconstituted with specified fluoride levels in the water for infants aged 0-12 months.

Fluoride level in water		0-2 months[a] 150 mL/kg/day mg F/kg/day			3-6 months[a] 120 mL/kg/day mg F/kg/day			7-12 months[a] 110 mL/kg/day mg F/kg/day		
		median	min	max	median	min	max	median	min	max
0.0 mg/L	MB:	0.009	0.005	0.020	0.007	0.004	0.016	0.006	0.004	0.015
	SB:	0.021	0.010	0.034	0.017	0.008	0.027	0.016	0.008	0.025
0.2 mg/L	MB:	0.039	0.040	0.050	0.031	0.030	0.040	0.028	0.030	0.040
	SB:	0.051	0.040	0.060	0.041	0.030	0.050	0.038	0.030	0.050
0.7 mg/L	MB:	0.114	0.110	0.130	0.091	0.090	0.100	0.083	0.080	0.090
	SB:	0.126	0.120	0.140	0.101	0.090	0.110	0.093	0.080	0.100
1.0 mg/L	MB:	0.159	0.160	0.170	0.127	0.120	0.140	0.116	0.110	0.120
	SB:	0.171	0.160	0.180	0.137	0.130	0.150	0.126	0.120	0.130

Notes: a) Only products suitable for infants of this age are included in calculations. Quantity of reconstituted infant formula consumed (mL/kg/day) based on Queensland Health formula feeding guidelines 2005.

Results

No samples tested were labelled as containing more than 17μg of fluoride per 100 kJ prior to reconstitution and results confirmed this. The median concentration of fluoride was 2.37 μg F/100kJ (min-max 1.21–7.20) or 0.46 μg F/g (0.24–1.20) in milk-based (cow and goat) formula powder and 5.15 μg F/100kJ (min-max 2.46–8.11) or 1.015 μg F/g (0.46 -1.60) in soy-based formula powder. When water with no fluoride was used for reconstitution, an average of 6.39 micrograms fluoride/100 mL (SD 2.27) for milk-based formula and 14.53 micrograms fluoride/100 mL (SD 8.66) for soy-based formula resulted. Comparing the findings of this study with the results of Silva and Reynolds, the average fluoride concentration of infant formula in Australia seems to have decreased, with milk-based formula containing about one-third the fluoride content, and soy-based formula about half the fluoride content found in 1996. It should be noted that this study included methodology to remove any fluoride impurities from the analytical reagent and therefore lower average fluoride levels might be expected compared to the Silva and Reynolds study.

The recommended volume of formula an infant consumes per day varies with age and weight.[13] Table 1 shows that average fluoride intake per kilogram body weight per day from infant formula varies considerably by age; 0.009 mg F/kd/day at age 0-2 months down to 0.006 mg F/kg/day at 6-12 months for milk-based products. The average contribution to daily fluoride intake from infant formula in milligrams of fluoride by average weight for age varied from 0.04 to 1.04 mg F/day for milk-based formula and 0.11 to 1.12 mg F/day for soy-based formula (Table 2). At levels of 0.7 mg/L of fluoride in water, estimates of intake identified in this study suggested intake exceeds guidance values but not UL values for all age groups. At water fluoride levels of 1 mg/L, estimates exceeded both guidance values and the UL values for all age groups.

Table 2: Estimated fluoride intake from milk-based (MB) and soy-based (SB) infant formula when reconstituted with specified fluoride levels in the water for infants aged 0-12 months.

Fluoride level in water		0-2 months (5.1 kg) mg F/day	3-6 months (7 kg) mg F/day	7-12 months (8.9 kg) mg F/day
0.0 mg/L	MB:	0.04	0.05	0.06
	SB:	0.11	0.12	0.14
0.2 mg/L	MB:	0.20	0.22	0.25
	SB:	0.26	0.29	0.34
0.7 mg/L	MB:	0.58	0.64	0.74
	SB:	0.64	0.71	0.83
1.0 mg/L	MB:	0.81[a]	0.89[a]	1.04[a]
	SB:	0.87[a]	0.96[a]	1.12[a]

Notes: a) Above UL (NHMRC 2005)

Discussion

Fluoride concentration in infant formula powder appears to meet Australian guidelines based on the samples used in this study, however infant formula products presented may not be representative of products available across Australia as samples were collected in Brisbane only. The results of this study are also limited to use of product as per manufacturer's recommendation and does not account for alternative use of products by consumers. Daily intake calculations are limited to similar total intake per day recommendation as those recommended by Queensland Health formula feeding guidelines and do not account for variations in feeding guidelines in other jurisdictions or personal preferences.

While none of the formula powders sampled demonstrated levels of fluoride in excess of FSANZ guidelines prior to reconstitution, calculations suggested that fluoride intake from powdered infant formula products, mainly soy-based, in areas with 1 mg/L of fluoride or above in the water may exceed upper limits and therefore may pose a risk of mild dental fluorosis.

This study aimed to estimate the amount of fluoride infants consumed from formula between birth and 12 months. This estimate is limited by the fact that National guidelines recommend breastfeeding exclusively from birth up to six months and that infants begin on solid food from six months of age with large variations in uptake.[3] Since no infant formula usage rates are available for Australia, only an estimate can be made from Australian breastfeeding data (exclusive and supplemented).[14] This would suggest that about 13% of infants at birth and up to 52% of infants at age six months are formula fed. However, given that the introduction of solids and drinking from cup also occurs around six months, 52% is likely to be an overestimate at six months of age and too much uncertainty exists to estimate formula use at 12 months of age.

While this research presents a more accurate picture of potential fluoride intake through infant formula consumption, it does not provide information on all sources of fluoride intake for these age groups. These findings are similar to overseas studies where the average intake for infants when formula is reconstituted with water at 1 mg/L fluoride have been estimated between 0.11 and 0.17 mg F/kg/day, with the highest intakes being recorded at two weeks of age.[11,15,16] Since studies have shown no evidence of benefit from fluoride until teeth erupt at about six months of age and evidence of mild fluorosis risk to baby teeth during this time has also been published, this study supports previous recommendations that water with 1 mg/L or more of fluoride not be used to reconstitute powdered infant formula, if a parent is concerned about a risk of their child experiencing mild fluorosis in baby teeth.[7]

While this study identifies that fluoride intake guidelines may be exceeded in some circumstances, there is insufficient evidence to confirm that the adequate and upper level intake values accurately depict the point at which the benefit of water fluoridation (protection from tooth decay) is outweighed by the risk (dental fluorosis) for children once the teeth begin to erupt.

Further research and in-depth analysis of all variables contributing to risk and benefit, for varying infant ages, would be required to more accurately predict AI and UL values.

With this in mind, it is hoped that these findings allow community members and health professionals to more accurately calculate the potential intake of fluoride from infant formula, and encourage further research to establish a stronger evidence base for guideline values.

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Fluoride Levels of Human Plasma and Breast Milk

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ABSTRACT

Objectives: The aim of this study was to determine the fluoride levels in breast milk and plasma of lactating mothers who regularly consumed drinking water with low levels of fluoride.

Methods: One hundred twenty five healthy mothers aged between 20-30 years old who had given birth within 5-7 days were included in the study. Besides being otherwise healthy, the primary selection criteria stipulated the absence of fluoride supplement consumption one month before delivery. Approximately 5 ml breast milk and 5 ml blood samples were obtained from each participating mother at a hospital setting, where the mothers were scheduled for a regular hospital diet. The blood samples were centrifuged in fluoride-free heparinized polyethylene tubes and stored at -18°C until measurements were made. Breast milk samples were directly refrigerated as with blood samples until measurements. The fluoride concentrations of milk and blood samples were assessed using an ion-selective fluoride electrode combined with an ion analyzer.

Results: The fluoride levels of the plasma and breast milk samples were measured as 0.017±0.011 ppm and 0.006±0.002 ppm, respectively. The fluoride concentration of plasma was significantly higher than that of breast milk ($P<.01$). Correlation analysis revealed a significant relation between the groups ($P<.01$).

Conclusion: A limited level of fluoride transmission from plasma to breast milk was detected. Nevertheless, a significant correlation between the fluoride concentrations of breast milk and plasma was evident. (Eur J Dent 2007;1:21-24)

Key words: Breast milk; Fluoride; Plasma.

INTRODUCTION

Since various forms of fluorides have met wide acceptance for use in the prevention of dental caries, the metabolism of fluoride is of considerable interest.[1-3] The human organism is exposed to fluoride in a number of ways. Ingestion of fluoride is accomplished through various foods; drinking water and fluoride containing products comprising dentifrices, mouth rinses, tablets, drops, etc.[4-6] Hard tissues are known to be the major sites of fluoride accumulation in the human body. Approximately 99% of the total body burden of fluoride is retained in bones and teeth, with the remainder distributed in highly-vascularized soft tissues.[4,5] Kidneys are the primary route for the removal of fluoride from the body. Other routes of fluoride excretion are sweat, feces, saliva and breast milk of lactating mothers.[4,5,7]

Breast milk is the major dietary intake of infants in the early stage of life. The level of fluoride in breast milk plays an important role as a fluoride supplement to the infant.[8] Conversely, the concentration of fluoride could be deemed critical regard-

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ing the potential dental fluorosis that may result from high concentrations of dietary fluoride.[9,10]

Plasma is the biological fluid into which fluoride must pass for its distribution elsewhere in the body as well as its elimination from the body. For these reasons, plasma is often referred to as the central compartment of the body.[6] Factors that include fluoride intake from various sources may affect plasma fluoride levels, and thus fluoride content of breast milk.

The aim of this pilot study was to determine the fluoride levels of breast milk and plasma of lactating mothers and the correlation between breast milk and plasma fluoride levels in mothers who regularly consume drinking water with low levels of fluoride.

MATERIALS AND METHODS

One hundred twenty five mothers aged between 20-30 years old with hospitalized newborns due to icterus neonatorum were included in the study. Signed consent was obtained from the participants after explanations regarding the study protocol. The human ethic committee of Selcuk University Experimental Research Center (SUDAM) approved this study (Approval No:2004-034).

Besides being otherwise healthy, the primary selection criteria stipulated the absence of fluoride supplement consumption one month before delivery. The participants regularly consumed drinking water from the same city supply which has been previously shown to contain low levels of fluoride (approx. 0.3 ppm).[11] The mothers consumed a regular hospital diet.

Milk and plasma samples were collected from lactating mothers within 5 to 7 days after delivery. For milk samples, the breast was swabbed with cotton wool and distilled water before milk collection. The mother was instructed to press the breast gently to facilitate collection of 5 ml of milk into a polyethylene tube. At the same appointment, 5 ml of blood was obtained and transferred into a fluoride-free heparinized polyethylene tube. Thereafter, the plasma was separated from the blood by centrifugation for 3 min at 3500 g. Milk and plasma samples were further stored at -18°C until analyses. Before fluoride measurements, the samples were thawed at room temperature.

To determine fluoride concentrations, equal volumes of TISAB II buffer (Orion Research, U.S.A.) was added into the samples. All samples were homogenized using magnetic stirrers throughout the measurements. An ion-selective electrode (Model 96-09, Orion Research, USA) was used in conjunction with a Model EA 910 ion analyzer (Orion Research, USA) to measure the fluoride concentrations of the breast milk and plasma samples.

Paired t test was used to determine the differences between fluoride concentration of breast milk and plasma. Pearson correlation analysis was used to assess any possible relationship between plasma and breast milk fluoride levels.[12]

RESULTS

The concentrations of fluoride in breast milk and plasma are presented in Table 1. The mean fluoride concentration of the plasma samples was 0.017±0.011 ppm (range 0.006-0.054 ppm). The mean fluoride concentration of the breast milk samples was 0.006±0.02 ppm (range 0.003-0.011 ppm).

Paired t test showed that the fluoride concentrations of plasma were significantly higher than those of the breast milk (P=.000). Pearson analysis revealed a significant correlation between the fluoride concentrations of breast milk and of plasma (P=.000). When a mother's plasma fluoride concentration was above (or below) the mean plasma fluoride level of the entire study group, the breast-milk fluoride levels were affected accordingly.

DISCUSSION

Several methods are used to determine fluoride levels in biologic tissues that include spectrophotometry,[13] gas chromatography,[14] capillary electrophoresis,[15] micro diffusion,[16] and ion analysis in conjunction with ion-selective electrodes.[17] As utilized in the present study, the most common procedure used to quantify free fluoride anion is the ion-selective electrode.[18]

The plasma fluoride concentration displays an increase along with fluoride intake. This increase is, however, attenuated due to distribution to the interstitial and intracellular fluid uptake by calcified tissues and renal excretion.[5] The literature contains a wide range (0.008-0.045 ppm) of reported normal plasma fluoride concentrations.[6,18] The diversity of values may have been due to the inclusion of fasting individuals as subjects in contrast to other studies employing non-fasting participants.[18] Certainly, other factors that include methodological variations as well as the fluoride levels of drinking-water consumed by subjects should have a strong impact on the reported values.[18] Li et al[19] reported a mean plasma fluoride concentration of 0.106±0.076 ppm in 127 subjects. In their study, the subjects were selected from a region with the drinking water fluoride concentra-

Table 1. Fluoride concentrations of breast milk and plasma.

	Min	Max	Mean±SD
Plasma (n=125)	0.006	0.054	0.017±0.011
Breast milk (n=125)	0.003	0.011	0.006±0.002

tions of 5.03 ppm. In the present study the mean plasma fluoride concentration was 0.017±0.011 ppm. Our finding corroborates those of Fejerskow et al[6] and World Health Organization (WHO).[18]

Breast milk possesses unique nutritional, biochemical, anti-infective and anti-allergic properties. As breast-fed infants obtain fluids almost exclusively from their mothers, breast milk represents an important way for delivering fluoride with certain levels to infants.[20] The level of fluoride in human milk has been a topic of investigation for many years. Medical literature contains a wide range for fluoride levels in breast milk. It is probable that problems with the analysis of fluoride have been contributory. According to the WHO,[18] the breast milk fluoride levels range from <0.002 to about 0.1 ppm, with most values being between 0.005-0.010 ppm. The mean breast milk fluoride concentrations obtained here in (0.006±0.002 ppm) are in line with the WHO.[18] Dabeka et al[8] showed that the concentration of fluoride in breast milk was related to the content of the drinking-water consumed by the mothers. The mean concentration of fluoride in breast milk obtained from 32 women consuming drinking water that contained < 0.16 ppm was 0.004 ppm, whereas breast milk obtained from 112 women consuming drinking water containing 1 ppm reportedly was 0.009 ppm.[8] Similar levels of fluoride concentrations of breast milk and colostrum (0.008 ppm) have been reported by Spak et al.[1] However, Spak et al[1] found no significant difference in breast milk fluoride concentrations of mothers living in areas with low and high drinking-water fluoride concentrations.

In the present study, the strict selection criteria which stipulated absence of recent use of fluoride supplements was a preventive measure to control variables that could interfere with the results. Additional limitations included selection of patients from a region with low drinking water fluoride levels (<0.3 ppm). In light of previous work,[8] however, it is apparent that the fluoride concentration of breast milk in mothers regularly consuming higher concentrations of fluoridated water is still within normal limits.

Theoretically, a limited transfer of fluoride from plasma to breast milk should occur.[21] The mechanism(s) responsible for the selective transfer of fluoride into breast tissue is obscure.[21] It is thought that a physiological plasma-milk barrier functions against to fluoride.[1,2,6] Despite high doses of supplementary fluoride administered to the mother, the child receives a maximum dose of only 0.2% of the mother's fluoride intake.[1,2] The results obtained in our study confirmed these conclusions. It should, however, be noted that the fluoride content transferred through breast milk is less than those present in cow's milk and in infant formulas, utilized as routine substitutes for breast milk. Rahul et al[3] found that fluoride concentrations of various commercially available infant milk formulations ranged from 1.95 ppm to 7.45 ppm and fluoride content of cow's milk samples was 0.12 ppm; values exceeding those of breast milk.

CONCLUSIONS

It has been verified by the positive correlation between plasma and breast-milk fluoride concentrations in subjects selected meticulously in terms of low drinking water fluoride levels. While the results of the present study confirm previous data regarding the difference between plasma and breast-milk fluoride concentrations, it is evident that more research, coupled with a wider study population is indicated to clarify the exact interactions between plasma fluoride levels and those of breast milk.

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Review

Molecular Mechanism of Action of Fluoride on Bone Cells

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ABSTRACT

Fluoride is an effective anabolic agent to increase spinal bone density by increasing bone formation, and at therapeutically relevant (i.e., micromolar) concentrations, it stimulates bone cell proliferation and activities in vitro and in vivo. However, the fluoride therapy of osteoporosis has been controversial, in large part because of a lack of consistent antifracture efficacy. However, information regarding the molecular mechanism of action of fluoride may improve its optimum and correct usage and may disclose potential targets for the development of new second generation drugs that might have a better efficacy and safety profile. Accordingly, this review will address the molecular mechanisms of the osteogenic action of fluoride. In this regard, we and other workers have proposed two competing models, both of which involve the mitogen activated protein kinase (MAPK) mitogenic signal transduction pathway. Our model involves a fluoride inhibition of a unique fluoride-sensitive phosphotyrosine phosphatase (PTP) in osteoblasts, which results in a sustained increase in the tyrosine phosphorylation level of the key signaling proteins of the MAPK mitogenic transduction pathway, leading to the potentiation of the bone cell proliferation initiated by growth factors. The competing model proposes that fluoride acts in coordination with aluminum to form fluoroaluminate, which activates a pertussis toxin–sensitive Gi/o protein on bone cell membrane, leading to an activation of cellular protein tyrosine kinases (PTKs), which in turn leads to increases in the tyrosine phosphorylation of signaling proteins of the MAPK mitogenic signal transduction pathway, ultimately leading to a stimulation of cell proliferation. A benefit of our model, but not the other model, is that it accounts for all the unique properties of the osteogenic action of fluoride. These include the low effective fluoride dose, the skeletal tissue specificity, the requirement of PTK-activating growth factors, the sensitivity to changes in medium phosphate concentration, the preference for undifferentiated osteoblasts, and the involvement of the MAPK. Unlike fluoride, the mitogenic action of fluoroaluminate is not specific for skeletal cells. Moreover, the mitogenic action of fluoroaluminate shows several important, different characteristics than that of fluoride. Thus, it is likely that our model of a fluoride-sensitive PTP represents the actual molecular mechanism of the osteogenic action of fluoride. (J Bone Miner Res 1998;13:1660–1667)

INTRODUCTION

CLINICAL STUDIES OF FLUORIDE in patients with osteoporosis have demonstrated that fluoride increases spinal bone density.[1] Past bone histomorphometric studies have indicated that the effect of fluoride to increase bone mass was due entirely to an increase in bone formation and not to a reduction in bone resorption, and that the stimulation of bone formation was mediated through an increase in the osteoblast number,[2,3] indicating that fluoride is an anabolic agent for bone cells and that the bone-forming effect of fluoride is mediated by an increase in osteoblast prolif-

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eration. However, although it is well recognized that the fluoride therapy is effective in increasing spinal bone density, the efficacy of the therapy in fracture reduction is highly controversial.[4–8] In this regard, fluoride therapy has an unfavorable benefit-to-risk profile, which is believed to be related to the high incorporation of fluoride in bone and to the fluoride-associated osteomalacia.[9] Because of this unfavorable efficacy profile, the fluoride therapy has not been generally regarded as a preferred therapy of osteoporosis. Consequently, in order for fluoride to be an effective therapy, this benefit-to-risk profile must be improved.

A knowledge of the mechanism of action of a drug is important for at least two reasons: it generally improves the optimum and correct usage of a drug, and it could disclose potential targets for the development of new second generation drugs, which might have a better efficacy and safety profile. We believe that both of these reasons are applicable to fluoride. For example, knowing how fluoride acts to stimulate bone formation should help to design an improved treatment regimen (i.e., intermittent or cyclic therapy) that could increase the benefit-to-risk profile of the therapy. Accordingly, intermittent therapy should reduce the risk for fluoride-induced osteomalacia and for the effect of fluoride to cause undesirably high levels of fluoride in bone. More importantly, information regarding the molecular mechanism of the signal transduction pathway of the osteogenic action of fluoride could provide screening targets for development of novel fluoride-like drugs with favorable efficacy and safety profiles. For example, if the fluoride-sensitive enzyme were available as a screening target, one could test small molecules for their ability to inhibit this enzyme and thereby stimulate cell proliferation. This could lead to the development of an orally active nontoxic fluoride mimic, which would specifically stimulate bone cell proliferation and bone formation.

Regarding the molecular mechanism of osteogenic action of fluoride, there are currently two competing models: one involves the inhibition of an osteoblastic fluoride-sensitive phosphotyrosine phosphatase (PTP) and the other involves the Gi/o protein-mediated activation of protein tyrosine kinases (PTKs). In this review, we will discuss the evidence for and against each model. To assist the assessment of these two models, a brief discussion of the mitogenic actions of fluoride on bone cells is included. Finally, we will also briefly discuss the other existing proposed models of fluoride's actions.

Fluoride ion stimulates osteoblast proliferation and activities in vitro

Farley et al.[10] provided the first in vitro evidence that fluoride acts directly on avian bone cells to stimulate cell proliferation in a biphasic fashion with the optimal mitogenic dose of ~10 μM levels, similar to the effective serum fluoride levels (i.e., basal level at 5–10 μM and peak level at 30 μM) in patients.[11] The bone cell mitogenic effects of fluoride was confirmed by a number of laboratories[12–20] and extended to bone cells of various species, including humans, as shown in Figure 1, which illustrated that fluoride at clinically relevant concentrations (5–30 μM) significantly increased the [^{3}H]thymidine incorporation and cell doubling in normal human bone cells in vitro. These in vitro mitogenic actions of fluoride are consistent with the past morphometric findings of a fluoride-dependent increase in bone cell number in vivo, indicating that the in vitro mitogenic action of fluoride may be clinically relevant. There is compelling evidence that fluoride at mitogenic, micromolar doses also stimulates several mature osteoblast activities, i.e., alkaline phosphatase expression,[10,13,14,21] collagen synthesis,[15,18] and osteocalcin synthesis[15,21] in monolayer bone cell cultures. Mitogenic doses of fluoride in vitro also stimulated transient calcium uptake[22,23] and sodium-dependent phosphate transport[24] in bone cells. Thus, it is generally believed that this stimulation of osteoblast proliferation and activities together leads to the increased bone formation.

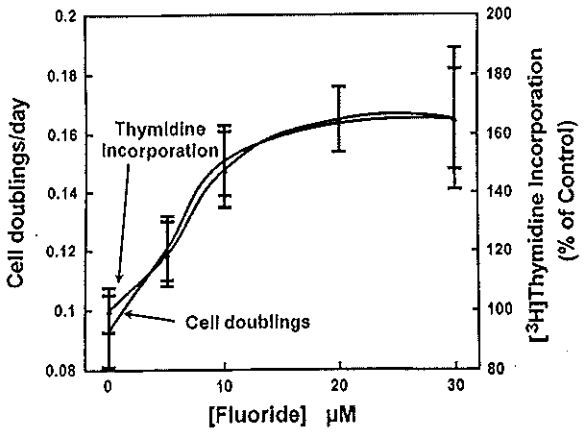

FIG. 1. Fluoride increases human bone cell proliferation in vitro. Normal human bone cells were isolated from the trabecular bone of femoral head samples obtained during hip replacement surgery with collagenase digestion. Cell proliferation was measured by [^{3}H]thymidine incorporation into DNA and by the number of cell population doublings per day. Each point is the mean and SEM of six replicates. (Adapted from Wergedal JE et al. Clin Orthop 233:274, 1988 with permission.)

Characteristics of in vitro mitogenic actions of fluoride

Several unique properties of the in vitro mitogenic actions of fluoride may be relevant to its molecular mechanism of the action. First, the mitogenic dose of fluoride is very low (micromolar) and at least two orders of magnitude lower than the doses of fluoride required for the effects on other biological systems (i.e., millimolar levels). Second, consistent with observations that the fluoride's in vivo anabolic effect is specific for skeletal tissues, the in vitro mitogenic activity of fluoride is also bone cell specific.[10,13] Third, there appears to be a requirement of a growth factor, such as insulin-like growth factor I or transforming growth factor β, for the in vitro bone cell mitogenic activity of fluoride.[19,25] Fourth, the mitogenic activity of fluoride is sensitive to changes in medium phosphate concentra-

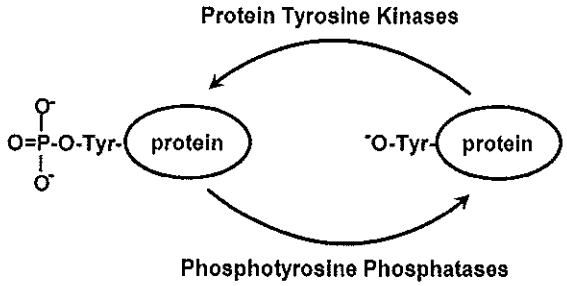

FIG. 2. Regulation of cellular tyrosyl protein phosphorylation. This figure illustrates schematically that the overall tyrosyl phosphorylation level of cellular proteins is controlled by two opposing enzymatic reactions: protein tyrosine kinases (PTKs) and phosphotyrosine phosphatases (PTPs).

tion.[25] Fifth, the fluoride ion acts primarily on osteoprogenitor cells and/or undifferentiated osteoblasts,[21,26,27] which synthesize an abundance of growth factors,[21,28] rather than to stimulate the proliferation of highly differentiated osteoblasts. Sixth, the bone cell mitogenic activity of fluoride involves increases in overall tyrosine phosphorylation of several cellular signaling proteins, including mitogen activated protein kinase (MAPK), in bone cells.[20,29–31] Accordingly, any attainable model for molecular mechanism of the mitogenic action of fluoride ion must account for these unique and intriguing properties.

A proposed molecular mechanism of anabolic actions of fluoride on bone cells

Regulation of cell proliferation is complex and involves multiple signal transduction pathways. However, there is now increasing compelling evidence that an increase in the overall tyrosyl phosphorylation level of key signaling proteins is essential for most, if not all, mitogenic signal transduction pathways. Figure 2 illustrates that the overall tyrosyl phosphorylation level of cellular proteins is determined by two important and opposing enzymatic reactions. Tyrosyl phosphorylation is catalyzed by PTKs, whereas the tyrosyl dephosphorylation is mediated by PTPs. Accordingly, an increase in the overall tyrosyl phosphorylation level of cellular proteins can be accomplished by a stimulation of the activity of PTKs, or by an inhibition of the activity of PTPs, or both.

With respect to the molecular mechanism of the osteogenic action of fluoride, we have advanced a model (shown in Figure 3A) that could account for the aforementioned unique properties of fluoride.[9,29] This model involves activation of the Ras-Raf-MAPK signal transduction pathway through increases in overall tyrosine phosphorylation levels of key signaling proteins via a fluoride-dependent inhibition of a unique osteoblastic PTP activity. In the Ras-Raf-MAPK pathway, the binding of a growth factor (e.g., insulin-like growth factor I) to its cell surface receptor activates the receptor's intrinsic PTK activity through autophosphorylation, which triggers a cascade of phosphorylation reactions, leading to the tyrosine phosphorylation of a number

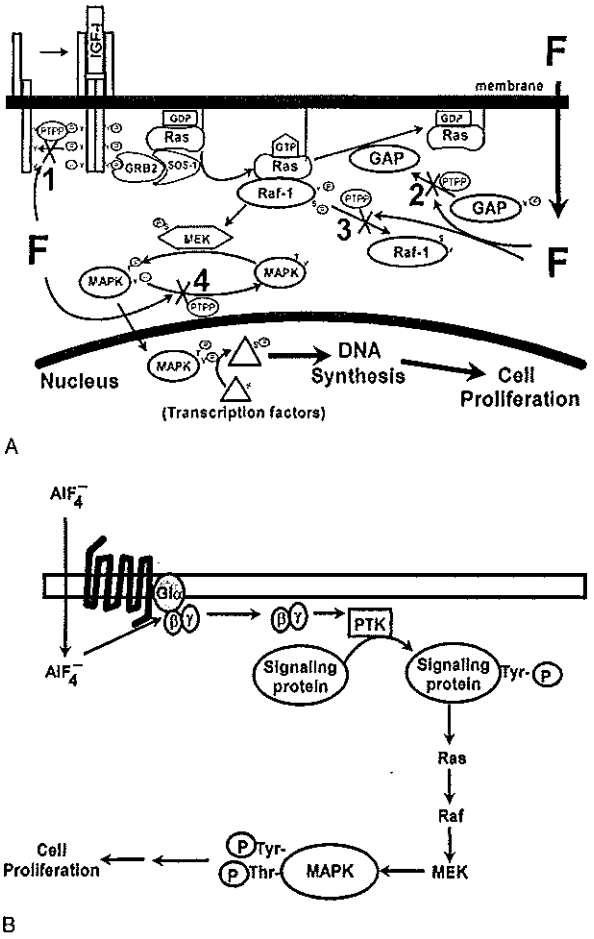

FIG. 3. Two competing proposed molecular mechanism for the bone cell mitogenic action of fluoride. (A) Our proposed model involving a fluoride inhibition of an osteoblastic fluoride-sensitive PTP. (Adapted from Libanati et al. in Osteoporosis, Marcus R, Feldman D, and Kelsey J (eds.) Academic Press, San Diego, CA, U.S.A., p. 1259, 1996 with permission.) (B) The competing model, which proposes the involvement of activation of Gi/o proteins by the fluoroaluminate complexes.

of cellular signaling proteins.[32,33] Accordingly, the activated growth factor receptor recruits the tyrosine phosphorylated docking proteins, such as Grb,[34] which targets Sos to the membrane to mediate the exchange of GDP to GTP on Ras.[35] The binding of GTP activates Ras, leading to activation and tyrosine phosphorylation of Raf.[36] Inactivation of Ras is mediated by rasGAP, which hydrolyzes the Ras-bound GTP to GDP.[35] Tyrosine phosphorylation of rasGAP leads to dissociation of Ras from rasGAP, thereby preventing the GTP hydrolysis and maintaining Ras in the activated state, leading to an activation of Raf.[36–38] Raf phosphorylates and activates MAPK/ERK protein kinase (MEK),[39] which phosphorylates MAPKs on both a threonine and a tyrosine residue,[39,40] resulting in their activation. The activated MAPKs migrate into the nucleus, phosphorylate, and activate a number of transcription factors and proto-oncogenes,[41,42] actions which collectively lead

to increased gene expression, DNA synthesis, cell proliferation, and/or differentiation.[39,42] This Ras-Raf-MAPK mitogenic pathway has at least four regulatory points: growth factor receptor, rasGAP, Raf, and MAPK (identified in Fig. 3A as 1–4, respectively), the activity of each of which is regulated by tyrosine phosphorylation. An overall increase in the tyrosine phosphorylation status leads to an activation of this pathway and cell proliferation and/or differentiation. We postulate that fluoride, upon its entry into bone cells, inhibits the activity of one or more unique fluoride-sensitive PTP(s), resulting in reduction of the dephosphorylation of one or more of these signaling proteins. As a consequence, their overall tyrosine phosphorylation level rises, which subsequently leads to the potentiation of osteoblast proliferation and/or activities initiated by the bone cell growth factor.

This model accounts for each of the aforementioned six unique characteristics of the fluoride-induced bone cell mitogenesis. First, osteoblasts contain a fluoride-sensitive acid phosphatase-like PTP.[29,43–46] Second, the fluoride-sensitive PTP activity is unique to osteoblasts and not found in other cells and tissues.[29] Thus, the effect of fluoride on this enzyme and the consequent anabolic effects should be specific for bone cells. Third, a fluoride-dependent inhibition of the dephosphorylation of tyrosine phosphorylated signaling proteins can effectively increase their overall phosphorylation level only when their basal phosphorylation has been increased in response to a PTK-stimulating growth factor. Thus, the optimal anabolic effect of fluoride on bone cells should require the presence of a bone cell growth factor (to increase the basal tyrosine phosphorylation level). Fourth, the fluoride ion may act, in coordination with divalent ions, as a transition state analog of phosphate.[47] Because transition state analogs of phosphate are potent inhibitors of PTPs, and because known transition state analogs of phosphate (e.g., vanadate and molybdate), at concentrations that inhibited an osteoblastic fluoride-sensitive PTP, also stimulated bone cell proliferation to the same extent as fluoride,[29,48] it may be speculated that the fluoride ion functions as a phosphate analog to promote bone cell mitogenic activity.[49–51] Accordingly, the finding that the bone cell mitogenic activity is sensitive to changes in phosphate concentration is consistent with the mechanism of a fluoride-dependent inhibition of an osteoblastic PTP to exert anabolic effects.[22,25] Fifth, osteoprogenitor cells and/or less differentiated bone cells are known to produce more growth factors than the highly differentiated bone cells,[21,28] and since the less differentiated osteoblasts contained higher amounts of the fluoride-sensitive PTP than the more differentiated osteoblasts,[27] it therefore is consistent with the premise that the less differentiated osteoprogenitor cells would be the preferred target cells for fluoride. Sixth, the findings that fluoride treatment increased steady-state tyrosine phosphorylation level of several key signaling proteins in bone cells in a sustained manner are entirely consistent with the contention that fluoride is an inhibitor of an osteoblastic PTP.[30,31]

Evidence supporting the proposed molecular mechanism of fluoride

Our model is supported by a large body of strong circumstantial evidence. First, a unique osteoblastic acid phosphatase-like PTP that is inhibited by clinically relevant doses of fluoride (apparent $K_i \sim 10–20$ μM) has been identified and purified.[29,43–46] Second, only osteoblasts, and not other cells and tissues (with the exception of the kidney), contain this fluoride-sensitive acid phosphatase-like PTP.[29] Third, mitogenic doses of fluoride increase the overall steady-state tyrosine phosphorylation level of a number of cellular proteins in human bone cells,[30] including key signaling proteins of the MAPK pathway, such as MAPK, Raf-1, and rasGAP.[31,52] Fourth, the mitogenic dose of fluoride is very low (10–100 μM) compared with its effect on other biological systems (i.e., millimolar levels), and is the same dose that inhibits the osteoblastic fluoride-sensitive PTP and that required for increased cellular tyrosine phosphorylation level.[29,30] Fifth, results of protein thiophosphorylation studies are consistent with an interpretation that fluoride acts to increase the tyrosine phosphorylation level through an inhibition of PTP rather than a stimulation of PTK.[29] Sixth, other inhibitors of PTPs (i.e., vanadate and molybdate), at concentrations that inhibit the fluoride-sensitive PTP, are mitogens for bone cells[29,44,51] and also increase the tyrosine phosphorylation level of cellular proteins, including MAPK.[30,31] Seventh, the time course of the effect on both bone cell mitogenesis and on tyrosine phosphorylation of cellular proteins exhibits a lag period and is sustained in nature, findings that are consistent with an inhibition of PTP and not compatible with a direct activation of tyrosine kinases.[30,31] Eighth, the time course of vanadate to increase the cellular tyrosine phosphorylation level is very similar to that of fluoride.[30,31] Ninth, like the bone cell mitogenic effect, the effect of fluoride to increase the tyrosine phosphorylation level of cellular protein in human bone cells requires the presence of a bone cell growth factor.[30] Tenth, the bone cell mitogenic action of PTP inhibitors, such as vanadate, like that of fluoride, also requires the presence of a bone cell mitogenic activity.[44] Consequently, we believe that these results taken together provide compelling, albeit circumstantial, support for our proposed model that the anabolic effects of fluoride are mediated through an inhibition of a fluoride-sensitive PTP to activate the MAPK signal transduction pathway in osteoblasts.

A competing model of mechanism of fluoride actions on bone cells

Bonjour and Caverzasio have made four observations in rodent bone cells that seemed to challenge the model shown in Fig. 3A. First, they reported that the bone cell mitogenic effects of fluoride on mouse MC3T3-E1 cells required the copresence of a micromolar dose of aluminum ion, in addition to a millimolar dose of fluoride,[52,53] suggesting the involvement of the fluoroaluminate complex (AlF_4^-), rather than the fluoride ion per se. Second, they found that a PTK inhibitor, genistein, inhibited the fluo-

ride-dependent phosphate transport and cell proliferation of UMR106 rat osteosarcoma cells.[20] Third, they were unable to observe a significant inhibition of PTP activities by AlF_4^- using artificial peptide substrates in extracts of the UMR106 rats and MC3T3-E1 bone cells.[20,53] Fourth, the bone cell mitogenic activity and tyrosine phosphorylation stimulating ability of AlF_4^- were completely blocked by pertussis toxin,[53] a presumed specific inhibitor of heterotrimeric Gi or Go proteins,[54] suggesting that a Gi/o protein may be involved. Accordingly, these investigators interpret these results to indicate that the osteogenic actions of AlF_4^- were mediated through the Gi/o-dependent activation of PTK activities, rather than an inhibition of a PTP activity.[20,52,53]

On the basis of these findings, Caverzasio and Bonjour have proposed a competing model of the osteogenic action of fluoride (shown in Fig. 3B), which also involves tyrosine phosphorylation and activation of a MAPK.[20,52,53] In contrast to the model shown in Fig. 3A, they postulate that fluoride forms complexes with the aluminum ion. Upon entering the cells, AlF_4^- acts directly on a specific Gi/o protein in bone cells, which subsequently leads to activation of one or more PTKs, resulting in the stimulation of tyrosine phosphorylation of several key signaling proteins, including Shc and $p42^{mapk}$ (or Erk 2). The activation of these signaling proteins is then responsible for the subsequent stimulation of cell proliferation and phosphate transport.[53]

While their data are consistent with their conclusions, we should also note that there are alternative interpretations to several of their findings that are compatible with the proposed model of a fluoride inhibition of a unique osteoblastic PTP (Fig. 3A). For example, genistein (and other PTK inhibitors) have been shown to inhibit cell proliferation induced by every test mitogen to date[55–57]; thus, it is not surprising that genistein inhibited the fluoride-induced bone cell proliferation. In this regard, we have also shown that PTK inhibitors blocked the stimulation of human bone cell proliferation by several known PTP inhibitors, such as phenylarsenic oxide and vanadate.[58] Accordingly, the abrogation of the effects by PTK inhibitors only confirms the concept that increased tyrosine phosphorylation is required for cell proliferation in general, but does not provide definitive proof of a direct involvement of activation of PTKs, as Caverzasio and Bonjour seemed to conclude (Fig. 3B). Moreover, we have shown convincingly that for fluoride to be mitogenic, there must first be a stimulation of mitogenesis by a growth factor whose effects are mediated by PTKs. Without a growth factor, fluoride and PTP inhibitors, such as vanadate, have no stimulatory effects.[19,25,46] The PTK inhibitor would be expected to lower the overall tyrosine phosphorylation level. We have shown that under such conditions, fluoride is incapable of stimulating cell proliferation, presumably because there is inadequate phosphorylated protein to preserve from dephosphorylation. Thus, the genistein results of Caverzasio and Bonjour are just as consistent with an inhibition of PTP as with the stimulation of PTK activities. Moreover, there is now abundant compelling evidence that pertussis toxin–sensitive G proteins can stimulate (or sometimes inhibit) the activity of protein phosphatases, including several PTPs[59–64] and PP2 A.[65] Most importantly, Sturgill and coworkers[63] have clearly demonstrated that pertussis toxin–sensitive G proteins inhibit the MAPK signaling pathway by inactivating Raf-1 through a direct activation of a PTP. Therefore, it would seem presumptuous to conclude a direct involvement of a Gi/o-dependent activation of PTKs, just because pertussis toxin blocked the mitogenic activity. Consequently, much additional work is required to definitively resolve the issue whether fluoride (or AlF_4^-) acts through an inhibition of PTPs or an activation of PTKs.

Regarding the lack of inhibition of the PTP activity by AlF_4^- in rodent bone cell extracts, we have clearly demonstrated that fluoride ion is a competitive inhibitor of the osteoblastic acid phosphatase-like PTP activity with an apparent K_i of 10–20 μM in both untransformed avian and human bone cells.[29,46] Competitive inhibition can be completely reversed with saturated substrate concentrations. Since the PTP assay in the studies of Caverzasio and Bonjour used a single, presumably saturated substrate concentration, and since we have evidence that the fluoride-sensitive acid phosphatase-like PTP accounts for only a very small portion of the total PTP activities in human bone cells (unpublished observations), it is likely that (especially with an inappropriate substrate) the inhibition of the fluoride-sensitive PTP activity was overlooked. Thus, one should be cautious about interpreting negative results. It may be premature at this time to completely rule out the possible involvement of an inhibition of a specific fluoride-sensitive PTP in the mechanism of fluoride or AlF_4^-.

The mitogenic activity of fluoride observed by Caverzasio and Bonjour has an absolute requirement for micromolar doses of aluminum ion. However, most workers[12–19,21–23] have observed the in vitro bone cell mitogenic activity of fluoride without the added aluminum ion. Thus, these findings do not support an absolute requirement for aluminum ion. Although one may argue that in past studies the amount of aluminum contamination in glassware, culture medium, and reagents might have been sufficient for an effect, it does not seem very likely since Bonjour and Caverzasio did not observe a mitogenic action unless 10–50 μM aluminum was added (assuming the culture supplies contained similar levels of aluminum contamination). More importantly, there is the lack of compelling clinical evidence for an absolute requirement of aluminum ion for the osteogenic action of the fluoride therapy in patients or animals.

An important issue with respect to the work of Bonjour and Caverzasio is that the fluoride concentration in their studies was at least an order of magnitude higher than the therapeutic serum fluoride level in patients[11] and also higher than the fluoride doses used in the previous studies.[12–19,21–23] It should also be pointed out that the mitogenic activity of fluoride is of biphasic nature[10,12–14,29–31]; while fluoride is mitogenic to bone cells at micromolar doses, it is an inhibitor to bone cell proliferation at millimolar levels. Accordingly, the requirement of a high fluoride concentration raises questions about the in vivo relevance of their findings with respect to the in vivo fluoride effects on bone formation. In addition, the model proposed by Bonjour et al. (Fig. 3B) does not account for the cell/

tissue specificity, the requirement of growth factor(s), or the sensitivity to phosphate of the bone cell actions of fluoride. Therefore, these apparent discrepancies must be resolved before a better understanding of the significance of their findings with respect to the osteogenic effects of fluoride can be attained.

We should also point out that AlF_4^- is not equivalent to the fluoride ion. Accordingly, there are significant differences between the responses to fluoride and those to AlF_4^-. For example, fluoride increased the overall level of tyrosine phosphorylation and activity of $p44^{mapk}$ (Erk1) in human bone cells,[31] whereas AlF increased the overall tyrosine phosphorylation level of $p42^{mapk}$ (Erk2) in rodent bone cells.[52,53] Moreover, because both the Al^- and AlF_4^- ion, each at micromolar levels, was able to stimulate human bone cell proliferation,[15,58] and because the Al^- ion and the fluoride ion each appears to act through a different mechanism to produce the respective bone cell mitogenic effects,[15] one cannot overlook the strong possibility that the mitogenic activity observed by Bonjour and coworkers was attributable to the Al^- (or AlF_4^-), rather than the fluoride ion. Thus, it is conceivable that the apparent discrepancies observed between Bonjour's and our laboratory could be because what they described in their studies is related to the mechanism of AlF_4^-, and not the fluoride ion, whereas what we observed is pertinent to the molecular mechanism of the fluoride ion. Alternatively, there is the remote possibility that the observed discrepancies were related to species difference (i.e., human vs. rodent). Further work is needed to resolve the apparent discrepancies observed between these two laboratories.

Other proposed mechanisms of fluoride action

In addition to the above two models, several other mechanisms of fluoride action on bone cells have also been proposed. Kawase and coworkers[66] have postulated that fluoride activates a heterotrimeric G protein to stimulate the protein kinase C activity, which subsequently leads to increased cell proliferation. Reed and coworkers[19] proposed that the mitogenic effect of fluoride be mediated by modulating bone cell sensitivity to transforming growth factor β. In addition, because fluoride treatment of bone cells triggered an acute increase in intracellular calcium levels,[22,23] and because increases in intracellular calcium have been associated with cell proliferation, it has been suggested that the increase in intracellular calcium may be, in part, involved in the mechanism of the bone cell mitogenic action of fluoride. More recently, Bourgoin et al.[67] presented evidence that 15–50 mM fluoride activated phospholipase D in human SaOs-2 osteosarcoma cells, which led to increased intracellular calcium, Arf kinase membrane relocation, and activation of protein kinase C. These effects were inhibited by the aluminum ion chelator deferoxamine. Accordingly, these investigators postulated that the action of AlF_4^- might be mediated through activation of phospholipase D through a G protein. Although each of these models is interesting and merits further investigation, none of these models can account for all of the aforementioned observations, such as the low (micromolar) effective dose of fluoride, the cell and tissue specificity of fluoride's mitogenic action, and/or the absolute requirements of an appropriate growth factor.

Concluding remarks

In summary, it is clear that, like the fluoride therapy, the molecular mechanism of fluoride action on bone cells is controversial. Current evidence strongly implicates the involvement of the activation of the Ras-Raf-MAPK signal transduction pathway through a fluoride-dependent increase in overall tyrosine phosphorylation status of key signaling proteins in this pathway. Increased tyrosine phosphorylation of these signaling proteins may be mediated either by an increase in PTK activities or by a decrease in PTP activities or both. However, unless there is evidence for a clinically relevant role for the observations seen with AlF_4^-, we must favor the possibility, on the basis of our own observations, that the most likely explanation for the increase in the tyrosine phosphorylation level of these signaling proteins of the MAPK pathway by a clinically relevant dose of fluoride is mediated through an inhibition of a specific osteoblastic fluoride-sensitive acid phosphatase-like PTP. Our current approach to settle this issue is to purify the putative fluoride-sensitive PTP, produce fluoride-sensitive PTP gene knockout mice, and determine if the knockout mouse has a phenotype similar to a fluoride-treated mouse. Regardless of the exact mechanism, these studies have clearly demonstrated that the bone cell mitogenic actions of fluoride are mediated through an enhancement of a mitogenic signal transduction pathway (i.e., the MAPK pathway) in bone cells. Accordingly, these observations raise the possibility that one may increase bone cell proliferation (and/or function) by enhancing a key signal transduction pathway, and as such these observations open up a new and exciting research area in which this key signal transduction pathway could be used as the screening target for discovery of new drugs for osteoporosis and related bone diseases.

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FLUORIDE

BACKGROUND

Fluoride is a normal constituent of the human body, involved in the mineralisation of both teeth and bones (Fairley et al 1983, Varughese & Moreno 1981). The fluoride concentration in bones and teeth is about 10,000 times that in body fluids and soft tissues (Bergmann & Bergmann 1991, 1995). Nearly 99% of the body's fluoride is bound strongly to calcified tissues. Fluoride in bone appears to exist in both rapidly- and slowly-exchangeable pools. Because of its role in the prevention of dental caries, fluoride has been classified as essential to human health (Bergmann & Bergmann 1991, FNB:IOM 1997).

Ingestion of fluoride in the pre-eruptive development of teeth has the effect of reducing caries due to uptake of fluoride by enamel crystallites and formation of fluorohydroxyapatite which is less soluble than hydroxyapatite (Brown et al 1977, Chow 1990). The post-eruptive effect on reducing caries is due to reduced acid production by bacteria and increased enamel remineralisation in acidogenic challenge (Bowden 1990, Hamilton 1990, Marquis 1995). Fluoride also has a unique ability to stimulate new bone formation and as such has been used as an experimental drug for the treatment of osteoporosis (Kleerekoper & Mendlovic 1993) although results have been variable depending on site assessed and the outcome measured (Kroger et al 1994, Riggs et al 1990, Sowers et al 1986, 1991).

Because of the low natural levels of fluoride in some water supplies and high levels of dental caries, many authorities worldwide, including Australia and New Zealand, have permitted, or instigated, fluoridation of water supplies. Although this has met some opposition, partly because of the potential health or dental effects that include fluorosis, the NHMRC concluded that a concentration of 1 mg/L secures most of the caries preventive effect available from fluoridated water, while maintaining minimal contribution of water fluoride to dental fluorosis in children and that there was no evidence of adverse health effects attributable to fluoride in communities exposed to a combination of fluoridated water (1 mg/L) and contemporary discretionary sources of fluoride (NHMRC 1991).

Not all Australian water supplies are fluoridated, notably those in parts of Queensland such as Brisbane. Concentrations in fluoridated areas are within the range identified by the NHMRC as safe and effective, varying from 0.6 mg/L in Darwin to 1.1 mg/L in Hobart. In New Zealand, the Ministry of Health (MOH) has recommended fluoridation of water supplies since the 1950s as the most effective and efficient way of preventing dental caries in communities receiving a reticulated water supply. In the Drinking Water Standards 2000, fluoridation is recommended at a level of 0.7–1.0 mg/L in drinking water. Around 85% of the New Zealand population is on what the government considers to be satisfactorily safe community water supplies in terms of fluoride content. Another 5% of the population are on community water supplies. Some of the larger centres without fluoridated water supplies currently are Whangarei, Tauranga, Wanganui, Napier, Nelson, Blenheim, Christchurch, Timaru and Oamaru.

The World Health Organization states in a review of chronic disease and diet that evidence that both locally applied and systemic fluoride are preventive for dental caries is convincing (WHO 2003).

One of the concerns expressed about fluoridation of the water supply relates to increasing rates of fluorosis in children seen in some communities over the same period as fluoridation has been practised. Dental fluorosis is a biomarker of over-exposure to fluoride among young children and results in a mottling of teeth. Recent research in Australia among children not exposed and exposed to water fluoridation indicated prevalences of 19% and 34%, respectively (Puzio et al 1993). However, Kumar et al (1989) have shown that the increases in fluorosis in other communities have been greater in areas with non-fluoridated water supplies and are likely to be due to increased intake of fluoride from supplements and ingestion from toothpaste and reconstituted infant formula (Osuji et al 1988, Pendrys & Stamm 1990).

Fluoride intake from most foods is low. Foods generally have concentrations well below 0.05 mg/100 g (Taves 1983). However, water in fluoridated areas, as well as beverages, teas, some marine fish and some infant formulas, especially those that are made or reconstituted with fluoridated water,

generally have higher concentrations. Other sources of fluoride include supplements and dental products. Water-soluble fluoride eg sodium fluoride, is nearly completely absorbed. The bioavailability may be reduced by the presence of calcium, magnesium, aluminium, iron or other cations. Absorbed fluoride is rapidly bound to the minerals in bones and teeth. Most of the non-retained or metabolic fluoride is excreted through the kidneys and the remainder via the intestines. In healthy young or middle-aged adults, about 50% of absorbed fluoride is retained and 50% excreted, but young children may retain as much as 80% (Eksterand et al 1994a,b).

Indicators used to assess the requirements for fluoride include prevalence of dental caries, measures of bone mineral content and fluoride balance studies.

1 mmol fluoride = 19 mg fluoride

RECOMMENDATIONS BY LIFE STAGE AND GENDER

Infants	**AI**	**Fluoride**
0–6 months	**0.01 mg/day**	
7–12 months	**0.50 mg/day**	

Rationale: The AI for 0–6 months was calculated by multiplying together the average intake of breast milk (0.78 L/day) and the average concentration of fluoride in breast milk of 0.013 mg/L (Dabeka et al 1986, FNB:IOM 1997) for mothers in areas with fluoridated water. Levels in formulas can vary widely depending on the concentration in the water used to reconstitute it. The AI for 0–6 months was based on extensive documentation about relationships between caries, water concentrations and fluoride intake (FNB:IOM 1997). A level of 0.05 mg/kg/day confers a high level of protection against caries and is not associated with unwanted health effects. Assuming a standard weight of 9 kg, this gives an AI of 0.5 mg/day. Infants living in non-fluoridated areas will not easily achieve the AI for fluoride, so supplements have been recommended based on life stage and level of water fluoridation.

Special note: Australian data have shown that prolonged consumption of infant formulas reconstituted with optimally-fluoridated water beyond 12 months of age could result in excessive amounts of fluoride being ingested during development of the enamel of the anterior permanent teeth and therefore may be a risk factor for fluorosis of these teeth (Silva & Reynolds 1996).

The majority of Australian/New Zealand infant formula manufacturers now control the concentration of fluoride. It is also possible to reduce concentrations by preparing formula using non-fluoridated water such as rain, filtered or spring water from non-volcanic areas in its preparation.

Supplements may be necessary for older infants in non-fluoridated areas. However, it is likely that many older infants and younger children are already ingesting 0.4–0.6 mg fluoride per day from foods, beverages and toothpaste alone (Burt 1992). A study of 60, 11–13 month old New Zealand infants (Chowdhury et al 1990) showed that total intake including fluoride from tablets and toothpastes ranged from 0.093 to 1.299 mg fluoride/day in fluoridated areas and from 0.039 to 0.720 mg fluoride/day in non-fluoridated areas. The fluoride from the diet (food and drink) ranged from 0.089 to 0.549 mg day in the fluoridated areas, and 0.038 to 0.314 mg day in the non-fluoridated areas.

Children & adolescents	AI	Fluoride
All		
1–3 yr	0.7 mg/day	
4–8 yr	1.0 mg/day	
Boys		
9–13 yr	2.0 mg/day	
14–18 yr	3.0 mg/day	
Girls		
9–13 yr	2.0 mg/day	
14–18 yr	3.0 mg/day	

Rationale: The AI for children is based on the requirement of 0.05 mg/kg body weight/day outlined above and adjusted for the standard body weights of 13 kg for 1–3 year olds, 22 kg for 4–8 year olds, 40 kg for 9–13 year olds, 64 kg for boys aged 14–18 years and 57 kg for 14–18 year-old girls. Supplements may be necessary for children in non-fluoridated areas, although the younger children (1–3 years) may already be getting much of their requirement from foods, beverages and toothpaste (Burt 1992).

Adults	AI	Fluoride
Men		
19–30 yr	4 mg/day	
31–50 yr	4 mg/day	
51–70 yr	4 mg/day	
>70 yr	4 mg/day	
Women		
19–30 yr	3 mg/day	
31–50 yr	3 mg/day	
51–70 yr	3 mg/day	
>70 yr	3 mg/day	

Rationale: The AI for adults is based on the requirement of 0.05 mg/kg body weight/day outlined above and adjusted for the standard body weights of 76 kg for men and 61 kg for women.

Pregnancy	AI	Fluoride
14–18 yr	3 mg/day	
19–30 yr	3 mg/day	
31–50 yr	3 mg/day	

Rationale: There is no evidence that requirements in pregnancy are greater than those of the non-pregnant woman.

Lactation	AI	Fluoride
14–18 yr	3 mg/day	
19–30 yr	3 mg/day	
31–50 yr	3 mg/day	

Rationale: There are no studies of the metabolism of fluoride in pregnancy. Fluoride concentrations in milk are very low and fairly insensitive to differences in the fluoride concentration of maternal drinking water. The AI is not greater than that of women in the non-pregnant, non-lactating state.

UPPER LEVEL OF INTAKE - FLUORIDE

Infants	
0–6 months	**0.7 mg/day**
7–12 months	**0.9 mg/day**
Children and adolescents	
1–3 yr	**1.3 mg/day**
4–8 yr	**2.2 mg/day**
9–13 yr	**10.0 mg/day**
14–18 yr	**10.0 mg/day**
Adults 19+ yr	
Men	**10.0 mg/day**
Women	**10.0 mg/day**
Pregnancy	
All ages	**10.0 mg/day**
Lactation	
All ages	**10.0 mg/day**

Rationale: The UL was set on the basis of moderate enamel fluorosis. A LOAEL of 0.10 mg/kg body weight for infants and children up to 8 years was set on the basis of community studies (Dean 1942, FNB:IOM 1997). A UF of 1 was applied, as the adverse effect is cosmetic rather than functional. For older children and adults, a NOAEL of 10 mg/day was derived based on data on the relationship between fluoride intake and skeletal fluorosis (FNB:IOM 1997, Leone et al 1954, 1955, McCauley & McClure 1954, Schlesinger et al 1956, Sowers et al 1986, Stevenson & Watson 1957). A UF of 1 was selected, as there are no signs of symptomatic skeletal fluorosis at this level of intake. No data exist to show increased susceptibility in pregnancy or lactation, so the same UL was adopted.

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COVER STORY

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THE SCIENCE AND PRACTICE OF CARIES PREVENTION

JOHN D.B. FEATHERSTONE, M.SC., PH.D.

A B S T R A C T

Background and Overview. Dental caries is a bacterially based disease. When it progresses, acid produced by bacterial action on dietary fermentable carbohydrates diffuses into the tooth and dissolves the carbonated hydroxyapatite mineral-a process called demineralization. Pathological factors including acid[illegible] bacteria (mutans streptococci a[illegible] lactobacilli), salivary dysfunct[illegible] dietary carbohydrates are relat[illegible] progression. Protective factors include salivary calcium, phos[illegible] proteins, salivary flow, fluorid[illegible] and antibacterial components [illegible] balance, prevent or reverse den[illegible]

Conclusions. Caries progression or reversal is determined by the balance between protective and pathologi[illegible]ctors. Fluoride, the key agent in h[illegible]ng caries, works primarily via topical mechanisms: inhibition of demineralization, enhancement of remineralization and inhibition of bacterial enzymes.

Clinical Implications. Fluoride in drinking water and in fluoride-containing products reduces caries via these topical mechanisms. Antibacterial therapy must he used to combat a high bacterial challenge. For practical caries management and prevention or reversal of dental caries, the sum of the preventive factors must outweigh the pathological factors.

Fluoride.. works via topical mechanisms:

Although the prevalence of dental caries in children has declined markedly over the last 20 years in most countries in the Western world, the disease continues to be a major problem for both adults and children everywhere.

The trends in caries in U.S. children during the last 30 years were recently summarized[1] on the basis of results of four national [illegible]roximately 75 percent of [illegible], about 70 percent of the [illegible]mately 25 percent of [illegible]r age range accounted for [illegible] By age 17 years, however, [illegible]80 percent of the caries.[1-6] These findings illustrate the need for management of caries by individual risk assessment and for measures more specifically directed to high-risk people and populations.

Although these prevalence rates still leave much to be desired, the overall caries prevalence in children has indeed declined in the United States. Smaller epidemiologic studies in recent years indicate, however, that the decline in caries has not continued during the 1990s and that it may have plateaued.[6]

Tooth eruption

The primary teeth

We don't usually think of a newborn as having teeth. However, at birth the crowns of the 20 "baby" or primary teeth are almost completely formed, and they are hidden from view in an infant's jawbones. The primary teeth gradually erupt through the gums during the first 2½ years of life.

The four front teeth—two upper and two lower—usually erupt first, beginning as early as six months after birth. Most children have a full set of primary teeth by the time they are 3 years old. The child's jaws continue to grow, making room for the permanent (adult) teeth that will begin to erupt at about age 6 years. Primary teeth begin to shed between ages 6 and 7 years. This process continues until about age 12 years.

The chart and photograph identify the names of the primary teeth and provide the approximate ages at which you can expect the teeth to erupt and shed.

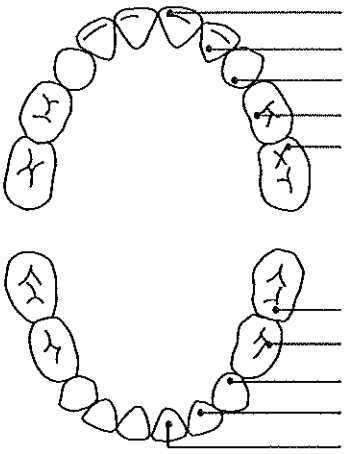
Primary teeth may be temporary, but they deserve good care. A child needs strong, healthy primary teeth not only to chew food easily, but to pronounce words properly.

This first set of teeth also holds a place in the jaw for the permanent teeth, which move into place as the primary teeth are shed. Primary teeth should be kept clean and healthy so that a child can remain free of cavities and oral pain. Infection from decayed primary teeth can damage the permanent teeth developing under them.

Parents and other caregivers may not realize that primary teeth are susceptible to decay as soon as they appear in the mouth. Tooth decay in infants and toddlers sometimes is called early childhood caries, baby bottle tooth decay or nursing mouth syndrome. This condition can destroy teeth. It occurs when a child's teeth are exposed frequently to sugary liquids for long periods.

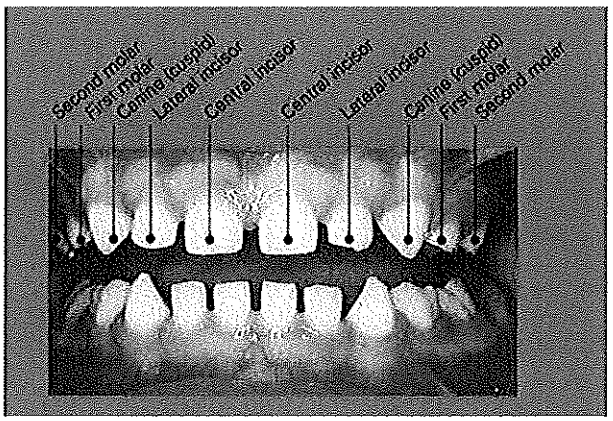
You can help reduce the risk of tooth decay. Never allow your infant or toddler to fall asleep with a bottle containing milk, formula, fruit juices or sweetened liquid. Don't dip a pacifier in sugar or honey. If your infant or toddler needs a comforter between regular feedings or at bedtime, give the child a clean pacifier recommended by your dentist or pediatrician.

Primary Teeth



Upper Teeth	Erupt	Shed
Central Incisor	8-12 Months	6-7 Years
Lateral Incisor	9-13 Months	7-8 Years
Canine (Cuspid)	16-22 Months	10-12 Years
First Molar	13-19 Months	9-11 Years
Second Molar	25-33 Months	10-12 Years

Lower Teeth	Erupt	Shed
Second Molar	23-31 Months	10-12 Years
First Molar	14-18 Months	9-11 Years
Canine (Cuspid)	17-23 Months	9-12 Years
Lateral Incisor	10-16 Months	7-8 Years
Central Incisor	6-10 Months	6-7 Years



Wipe your child's gums with a wet washcloth or a clean gauze pad after each feeding. Begin brushing your child's teeth with a little water as soon as the first tooth appears. Supervise toothbrushing to make sure that children older than 2 years use only a pea-sized amount of fluoride toothpaste and avoid swallowing it. Children should be taught to spit out remaining toothpaste and rinse with water after brushing. ■

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Chronic Fluoride Toxicity: Dental Fluorosis

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Abstract

Dental fluorosis occurs as a result of excess fluoride ingestion during tooth formation. Enamel fluorosis and primary dentin fluorosis can only occur when teeth are forming, and therefore fluoride exposure (as it relates to dental fluorosis) occurs during childhood. In the permanent dentition, this would begin with the lower incisors, which complete mineralization at approximately 2–3 years of age, and end after mineralization of the third molars. The white opaque appearance of fluorosed enamel is caused by a hypomineralized enamel subsurface; with more severe dental fluorosis, pitting and a loss of the enamel surface occurs, leading to secondary staining (appearing as a brown color). Many of the changes caused by fluoride are related to cell/matrix/mineral interactions as the teeth are forming. At the early maturation stage, the relative quantity of amelogenin protein is increased in fluorosed enamel in a dose-related manner. This appears to result from a delay in the removal of amelogenins as the enamel matures. In vitro, when fluoride is incorporated into the mineral, more protein binds to the forming mineral, and protein removal by proteinases is delayed. This suggests that altered protein/mineral interactions are in part responsible for retention of amelogenins and the resultant hypomineralization that occurs in fluorosed enamel. Fluoride also appears to enhance mineral precipitation in forming teeth, resulting in hypermineralized bands of enamel, which are then followed by hypomineralized bands. Enhanced mineral precipitation with local increases in matrix acidity may affect maturation stage ameloblast modulation, potentially explaining the doserelated decrease in cycles of ameloblast modulation from ruffleended to smooth-ended cells that occur with fluoride exposure in rodents. Specific cellular effects of fluoride have been implicated, but more research is needed to determine which of these changes are relevant to the formation of fluorosed teeth. As further studies are done, we will better understand the mechanisms responsible for dental fluorosis.

Excess fluoride ingestion results in dental fluorosis. The mechanisms affected by longterm chronic exposure to low levels of fluoride are likely to differ from those affected by acute exposures to high levels of fluoride [1–3]. Some mechanisms affected by lower chronic fluoride levels, resulting in enamel fluorosis, are likely to be specific to this uniquely mineralizing tissue, while others may also affect other cells and tissues.

Enamel fluorosis refers to fluoride-related al-terations in enamel, which occur duringenamel development. These alterations become more severe with increasing fluoride intake, and time of exposure. The severity of fluorosis is related to the concentration of fluoride in the plasma, considered to be in equilibrium with the tissue fluid that bathes the enamel organ [4, 5]. Plasma fluoride levels are influenced by many factors, including total fluoride intake, type of intake (i.e. ingested vs. inhaled), renal function, rate of bone metabolism, metabolic

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activity, etc. [6]. In addition to these variables, genetic factors have been shown to dictate the severity of enamel fluorosis in mice [7].

In humans, plasma fluoride concentrations resulting from long-term ingestion of 1–10 ppm fluoride in the drinking water range from 1 to 10 μmol/l. Fluorotic changes can be obtained in incisors of rodents drinking water containing 25–100 ppm fluoride; these doses also elevate plasma fluoride levels to 3-10 μmol/l, similar to those found to cause fluorosis in humans. A complicating factor in assessing the exact dose, or determining the stages of enamel formation most sensitive to fluoride, is that fluoride incorporated into bone is gradually released by continuous bone remodeling [5, 8]. Levels of plasma fluoride as low as 1.5 μmol/l (resulting from fluoride release from bone) are still capable of inducing mild enamel fluorosis in the rat incisor after the initial exposure ends [4, 8].

The effects of chronic fluoride exposure have also been linked to effects on other tissues and systems [9]. However, in this chapter, we will focus primarily on the effects of fluoride on tooth development. The largest body of research has investigated the effects of fluoride on enamel formation, with much less known about the potential effects of fluoride on dentin formation. Therefore, most of the focus will be on enamel fluorosis. The sections of this chapter comprise:

1. Clinical manifestation, treatment and prevention of dental fluorosis
2. Etiology and prevalence of dental fluorosis
3. Pathology, pathogenesis and mechanism of dental fluorosis

Clinical Manifestation, Treatment and Prevention of Dental Fluorosis

Clinical Manifestations of Dental Fluorosis

Clinically, mild cases of dental fluorosis are characterized by a white opaque appearance of the enamel, caused by increased subsurface porosity (fig. 1). The earliest sign is a change in color, showing many thin white horizontal lines running across the surfaces of the teeth, with white opacities at the newly erupted incisal end. The white lines run along the 'perikymata', a term referring to transverse ridges on the surface of the tooth, which correspond to the incremental lines in the enamel known as Striae of Retzius [10, 11].

At higher levels of fluoride exposure, the white lines in the enamel become more and more defined and thicker. Some patchy cloudy areas and thick opaque bands also appear on the involved teeth. With increased dental fluorosis, the entire tooth can be chalky white and lose transparency [10, 12]. With higher fluoride doses or prolonged exposure, deeper layers of enamel are affected; the enamel becomes less well mineralized. Damage to the enamel surface occurs in patients with moderate-to-severe degrees of enamel fluorosis. Teeth can erupt with pits, with additional pitting occurring with posteruptive enamel fracture.

In the individuals with moderate dental fluorosis, yellow to light brown staining is observed in the areas of enamel damage. In very severe cases, the enamel is porous, poorly mineralized, stains brown, and contains relatively less mineral and more proteins than sound enamel. Severely fluorosed enamel can easily chip posteruptively during normal mechanical use [13, 14]. Although teeth with mild dental fluorosis may be more resistant to dental decay because of the higher levels of fluoride contained in the enamel surface, severely fluorosed teeth are more susceptible to decay, most likely because of the uneven surface or loss of the outer protective layer [15].

Fluorosis Indices

In 1942, H.T. Dean developed an index to describe and diagnosis enamel fluorosis [16, 17]. He scored the fluorotic teeth into 6 categories according to their clinical manifestations, including normal teeth, which were given a score of 0 (table 1). Using this index, Dean [17, 18] determined the 'optimal' concentration of fluoride in drinking water (1 ppm), where caries incidence decreased and with a minimal level of dental fluorosis.

This classification is still the 'gold standard', though other indices have been developed– including the widely used Thylstrup and Fejerskov Fluorosis Index (TFI) [19], which has an expanded range for the more severe forms of dental fluorosis. This index is a 10-point classification system to characterize dental fluorosis affecting bucal/lingual and occlusal surfaces and correlates visual assessment with polarized and light microscopic analysis [19]. Dean's index is expanded to include: mild (TFI = 1–3), moderate (TFI = 4–5) and severe (TFI = 6–9) [19].

Treatment of Dental Fluorosis

The treatments for fluorotic teeth are limited. For the mildest forms of fluorosis (TFI 1, 2) bleaching can be recommended. Treatments for moderate dental fluorosis include microabrasion, where the outer affected layer of enamel is abraded from the tooth surface in an acidic environment. Composite restorations combined with microabrasion or application of aesthetic veneers can be used for the patients with TFI ≥5, while for the cases with TFI 8–9, prosthetic crowns may be necessary [19].

Prevention of Dental Fluorosis

Dental fluorosis can be limited or prevented by following the 'recommended limits for fluoride exposure', suggested by US Environmental Protection Agency (USEPA) [20]. The reference dose suggested by USEPA is 0.06 mg fluoride/kg/ day, which is the estimate of daily exposure that is likely to be without any appreciable risk of deleterious effects (any degrees of dental fluorosis) during a lifetime [20].

Specific guidelines for different ages (table 2) were published by the US Food and Nutrition Board of the Institute of Medicine in 1997, recommending total daily fluoride intakes [21]. In this guideline, the suggested total daily exposure dosage for infants younger than 6 months of age of 0.01 mg fluoride/day in all drinks and food is lower than the USEPA recommended reference dose. These guidelines suggest greater attention should be given to the total fluoride intake of infants from water used to dilute infant formulas, foods and other supplement sources.

Etiology and Prevalence of Dental Fluorosis

There are multiple sources of fluoride and all have the potential to cause dental fluorosis – including natural fluoride, artificial or added fluoride in drinking water and dental products, as well as occupation-related exposures.

Natural Sources of Fluoride Causing Dental Fluorosis

Dental fluorosis resulting from high fluoride levels in underground water is an issue in specific regions of the world. Fluoride can exist in an ionized form in ground waters, and in areas where the soil lacks calcium – such as occurs in areas with high levels of granite or gneiss – relatively high fluoride levels are detected in groundwater. When the level of fluoride is above 1.5 mg/l (1.5 ppm) in drinking water, dental fluorosis can occur. In some parts of Africa, China, the Middle East and southern Asia (India, Sri Lanka), as well as some areas in the Americas and Japan, high concentrations of ionic fluoride have been found in

ground waters, vegetables, fruit, tea and other crops, although drinking water is usually the major source of the daily fluoride intake [22]. The atmosphere in these areas may have high levels of fluoride from dust in areas with fluoride-containing soils and gas, released from industries, underground coal fires and volcanic activities [22].

In the USA, approximately 10 million people are exposed to naturally fluoridated public water. In 1993, it was reported that 6.7 million people drank water with fluoride concentrations ≤1.2 mg/l, 1.4 million drank water with 1.3–1.9 mg/l fluoride, 1.4 million drank water with fluoride between 2.0 and 3.9 mg/l and 200,000 people ingested water with fluoride concentrations ≥4.0 mg/l [16]. Some areas have extremely high concentrations of fluoride in drinking water – such as in Colorado (11.2 mg/l), Oklahoma (12.0 mg/l), New Mexico (13.0 mg/l) and Idaho (15.9 mg/l) [9] – though water with levels higher that those recommended by the USEPA are monitored and are not used for human consumption.

Additional Sources of Fluoride Associated with Dental Fluorosis

Two primary sources have been identified as being potentially responsible for the prevalence of dental fluorosis: fluoride in drinking water and fluoride-containing dental products (including fluoride supplements). Since 1945, fluoride has been used as a supplement in many public drinking water systems to control dental decay [23]. In 2000, approximately 162 million people (65.8% of the population served by public water systems) received water that contained fluoride ranging from 0.7 to 1.2 mg/l (usually 1 mg/l), depending on the local climate. The level of fluoridation is lower in high-temperature areas as people usually drink more water. The fluoridation of public drinking water has significantly decreased the incidence of dental decay at a relatively low cost. In the studies by Dean and colleagues completed in the 1930s, the risk of dental fluorosis at 1 ppm fluoride in drinking water was extremely low, particularly in relation to the impact of fluoride on dental caries (fig. 2) [24]. Following these studies, water fluoridation was considered by the US Centers for Disease Control to be 1 of the 10 great public health achievements in the 20th century [25].

However, as fluoride has become more widely used in dental products (toothpastes, mouth rinses, fluoride supplements) and been incorporated into food sources (via fluoridated water), multiple sources of fluoride exposure are now related to the reported increase in the incidence of dental fluorosis. Even a small 'pea-sized' amount of toothpaste containing 1,450 ppm fluoride, would contain approximately 0.36–0.72 mg fluoride, which if consumed twice a day could contribute to fluoride levels that would increase the risk of dental fluorosis in children [26]. In the USA, the prevalence of dental fluorosis appears to be increasing. In children aged 15–17 years, the 1999–2004 National Health and Nutrition Examination Survey (NHANES) found 40.6% had very mild or greater enamel fluorosis, up from 22.6% in the 1986–1987 study (fig. 3) [27].

The incidence of very mild and greater fluorosis in persons aged 6–39 years was 19.79% in white non-Hispanics, 32.88% in black non-Hispanics, and 25.8% in Hispanics (table 3). The increased prevalence of fluorosis in black non-Hispanics may suggest a genetic influence on fluorosis susceptibility.

Pathology, Pathogenesis and Mechanism of Dental Fluorosis

The primary pathological finding of fluorosed enamel is a subsurface porosity, along with hyper and hypomineralized bands within the forming enamel (fig. 4) [28–34]. Fluoride can also result in mineralization-related effects on dentin formation.

Severely fluorosed human dentin is characterized by a highly mineralized sclerotic background pattern, scattered with hypomineralized porous lesions primarily in the subsurface area. Scanning electron microscope images show dentin tubules with an irregular distribution and narrow and disrupted lumina, rather than the regular appearing lumina seen in normal dentin [35].

The pathogenesis of dental fluorosis is related to physiological conditions, including body weight, rate of skeletal growth and remodeling, nutrition, and renal function [36–38]. Bone is a reservoir of fluoride, as fluoride is incorporated in the forming apatite crystals, and this ion can also be released from these crystals as bone remodels. Therefore, rapid bone growth, as occurs in the growing child, will remove fluoride from the blood stream, possibly reducing the risk of dental fluorosis by lowering serum fluoride levels [8, 39]. Nutrition is also important for controlling the serum level of fluoride, as ions such as calcium, magnesium and aluminum can reduce the bioavailability of fluoride. A deficiency in these ions in food can also affect (enhance) fluoride up take [40].

Genetic background appears to have role in the pathogenesis of dental fluorosis. This may be the reason why in human populations, individuals drinking water with similar fluoride contents have a wide range of severity of dental fluorosis (fig. 2). Evidence for a genetic component to fluoride susceptibility comes from work by Everett et al. [7], which tested 12 different inbred mouse strains to compare their susceptibility to fluoride. Mouse teeth have been found to be an excellent model for human tooth formation, and in Everett's study, they found that some mouse strains were highly susceptible to fluoride related dental fluorosis, while other strains were highly fluorosis resistant. They concluded that there is a genetic component to dental fluorosis susceptibility [41, 42].

Stages of Tooth Formation and Stage-Specific Effects of Chronic Fluoride Exposure

Fluoride is a single highly electronegative ion that interacts with the cells and matrix at the different stages of enamel formation in relation to fluoride dose and time of exposure. Tooth enamel development can be divided into 4 major stages: pre-secretory, secretory, transition and maturation stages, all with unique properties that affect fluoride susceptibility. Most of the studies of the mechanisms of fluoride in forming fluorosed enamel have used the rodent incisor or molars as a model, as it is not possible to do similar studies using human teeth. The rodent incisor is a continuously erupting tooth, with all stages of enamel formation present in each tooth, whereas the molar is a rooted tooth, which begins formation in utero. As previously mentioned, though rodents require the ingestion of much higher levels of fluoride in the drinking water (10–20 times) as compared to humans, the serum levels at which fluorosis is formed in rodents and humans is similar.

Pre-secretory ameloblasts differentiate into secretory ameloblasts after the dentin matrix begins to mineralize. The pre-secretory ameloblasts and overlying cells of the enamel, including the enamel knot, are thought to influence the tooth morphogenesis. However, there is no evidence that exposure of developing teeth to physiological levels of fluoride in vivo [43] and in organ culture [44–47] affects tooth morphogenesis. Even in teeth with severe fluorosis, the size and form of the teeth are not changed [48].

As the pre-ameloblasts differentiate to secretory ameloblasts, they begin to secrete enamel matrix proteins, and lay down a thin layer of aprismatic enamel deposited against mantle dentin. As the secretory ameloblast Tomes' processes form, the inner enamel layer, which constitutes the bulk of enamel, begins to be laid down. This enamel matrix consists of prismatic enamel with rod (or prisms) and interrod structures (interprismatic enamel) formed by the Tomes' processes of fully differentiated secretory ameloblasts. These cells secrete

matrix protein (predominantly amelogenins) into the enamel space through which thin but long enamel crystals grow preferentially in length in the wake of the retreating cells.

Secretory stage ameloblasts exposed to high chronic levels of fluoride have a somewhat disrupted morphology and increased numbers of vacuoles at the apical border. Chronic exposure to fluoride in drinking water or repeated injections of moderate fluoride doses reduces the thickness of enamel by about 10% [43, 49]. Although this suggests that chronic exposure to fluoride reduces biosynthesis of matrix by secretory ameloblasts, there is no evidence to support this [1, 50, 51]. Instead, the small reduction in enamel thickness may be attributed to a limited disruption of vesicular transport in fluorotic secretory ameloblasts and subsequent intracellular degradation of a minor portion of the matrix by the lysosomal system [52–54].

At the end of secretion, the ameloblasts lose their Tomes' process and deposit a final layer of aprismatic enamel with small crystals. The cells transform via a short transitional stage, where enamel matrix proteins undergo rapid proteolysis, leaving the porous enamel matrix characteristic of this transition stage.

The late secretory-transitional cell stage ameloblasts appear to be more sensitive to fluoride than early and fully secretory ameloblasts. In hamster molar tooth germs, a dose of 4.5 mg/kg fluoride induces the late secretory to transitional cells, but not early secretory ameloblasts to detach occasionally from the surface and form subameloblastic cysts. The enamel below the cysts under late secretory ameloblasts will give rise to the shallow occlusal pits, often seen in severely fluorosed teeth in various species [48, 55–61]. This stage of development is likely also to be associated with the formation of accentuated perikymata that is clinically the first sign of enamel fluorosis.

In the maturation stage, the ameloblasts modulate cyclically from cells with a smooth-ended to a ruffleended distal membrane, the latter with characteristics of resorbing cells. During this modulation, matrix proteins continue to be removed from the extracellular space, and mineralization increases to form a fully mineralized enamel matrix. Amelogenin proteins are retained in the fluorosed rat enamel matrix at this stage of enamel formation [51, 62].

Maturation ameloblasts of adult rat incisors [43] are shorter, and fluorotic enamel organs have a disrupted maturation ameloblast modulation [43, 63, 64]. The first modulation bands that disappear during fluoride exposure are the most incisal smoothended ameloblasts. At prolonged exposure other smooth-ended bands disappear one by one in an incisal to apical direction [63]. In addition to changes in modulation, fluoride also reduces the cyclic uptake of ^{45}Ca labeling in a similar pattern [63]. When fluoride exposure is discontinued, smooth-ended bands reappear starting from the youngest most apical part towards older more incisal bands. This suggests that the fluoride effects on ameloblast modulation are reversible, and that the young modulating cells recover more rapidly than older ameloblasts. After eruption, the enamel is exposed to mineral ions of the oral fluids, including fluoride, which can influence the composition of the outer layers of enamel.

Direct Effects of Fluoride on Ameloblasts

Ameloblasts and tooth organs exposed to high (millimolar) levels of fluoride in vitro, which would be much greater than the micromolar levels of fluoride found in the plasma carrying fluoride ions to tooth organs in vivo, show many alterations. These include changes in the structure of early secretory ameloblasts, reduced protein synthesis, altered cell proliferation, apoptosis, stress-related protein upregulation and elevation of F-actin [65–68]. However, some of these same changes are not readily apparent in vivo, and therefore, the effects of fluoride when examined in culture, must be carefully analyzed for biological relevance.

However, there are in vitro data indicating that ameloblasts can be sensitive to low levels of fluoride. Human primary enamel organ epithelial cells grown in culture show that exposure to fluoride levels as low as 5 μmol/l results in reduced expression of the secretory stage matrix metalloproteinase 20 (MMP-20) [69], mediated by JNK/ c-Jun signaling [70]. These results suggest that fluoride may have specific effects on ameloblast differentiation mediated through MAP-kinase signaling.

Rodent studies have shown that ingestion of fluoride alters the number of bands of smooth ended ameloblasts and their rate of modulation in the maturation stage ameloblasts [43, 63]. However, there is currently no evidence to determine whether these changes in maturation stage ameloblast modulation are a direct effect of fluoride, or more likely, in response to matrixmediated alterations related to fluoride exposure to the developing enamel matrix.

At extremely high levels of ingested fluoride (150 ppm) in the drinking water, ameloblasts have been shown to exhibit apoptosis and endoplasmic reticulum stress responses [66]; however, at lower levels (75 ppm) these effects were not noted. Further studies at lower fluoride levels will need to be done to determine whether this is a potential mechanism relevant to chronic fluoride toxicity in humans.

Fluoride-Related Alterations of the Forming Enamel Matrix May Indirectly Affect Ameloblast Function

The extracellular enamel matrix proteins include amelogenins, ameloblastin and enamelin, all of which support and modulate enamel crystal formation [71]. Amelogenin is the chief structural protein constituting 90–95% of total proteins in the enamel protein matrix [72]. Amelogenin and the other matrix proteins are hydrolyzed by matrix proteinases as enamel forms, allowing replacement of the protein matrix with an organized hydroxyapatite structure. MMP-20 is the proteinase primarily responsible for the initial hydrolysis of amelogenins in the secretory enamel matrix, while kallikrein 4 (KLK4) is the predominant proteinase in the transition/maturation stage [73, 74].

An analysis of proteolytic activity in enamel matrix, isolated from secretory and maturation stage rat enamel, showed a significantly reduced activity in early maturation stage enamel isolated from rats ingesting 100 ppm fluoride (5–10 μm serum fluoride), as compared to control maturation enamel [75]. This effect of fluoride ingestion in decreasing matrix proteinase activity correlates to an increased retention of amelogenin proteins in maturation stage fluorosed enamel in a dosedependent manner (fig. 5). Matrix proteins disappear from nonfluorosed enamel in the maturation stage, but are retained in fluorosed enamel, with increased retention at higher levels of ingested fluoride [49, 51].

This retention of amelogenin proteins could delay final mineralization of the enamel matrix, contributing to subsurface hypomineralization characteristic of fluorosed enamel. The reason for this retention of amelogenins is most likely related to altered proteolytic activity in the fluorosed enamel matrix.

Reduced Proteolytic Activity May Be due to the Effects of Fluoride Incorporation into Growing Enamel Crystals

Crystals in sound enamel are long, and the dynamics of enamel crystal growth, size of the crystals and their shape are well controlled by matrix proteins during enamel formation [76–78]. Some studies report that crystals isolated from fluorosed enamel have a significantly greater diameter than crystals in sound enamel, as determined by high-resolution electron microscopy [79], X-ray diffraction of powdered enamel samples [80] or scanning microscopy of fractured inner enamel specimens [81]. Some organ culture studies have shown large flattened hexagonal crystals mixed with many small irregularly shaped crystals

in hypermineralized areas [82, 83]. However, other studies reported no differences between fluorotic and normal human crystals [28, 84].

There is, however, no doubt that the fluoride content of crystals in fluorosed enamel is greater than that of normal enamel. Fluoride substitutes for hydroxyl groups in enamel carbonated hydroxyapatite crystals, altering the crystalline structures and surface characteristics. To determine whether an increased fluoride content of the apatite crystals could affect matrix/proteinase interactions, we measured the binding of recombinant human amelogenin to synthetic carbonated hydroxyapatite crystals.

The initial rate of amelogenin binding and the total amount of amelogenin bound to fluoride-containing carbonated hydroxyapatite was greater than that in the control carbonated hydroxyapatite [85]. These results suggest that fluoride incorporation into the crystal lattice alters the crystal surface to enhance amelogenin binding, potentially contributing to the increased amount of amelogenin and the inhibition of crystal growth in fluorosed enamel.

In further investigation of the role of fluoride incorporation into apatite on amelogenin processing, we characterized hydrolysis of amelogenins bound to fluoride-containing apatites by recombinant MMP-20 or KLK-4. When fluoride was in solution, amelogenin hydrolysis by MMP-20 was reduced only at 1,000 ppm (52 mm, which is far higher than physiological levels of fluoride in enamel fluids). However, incorporation of fluoride into apatite significantly delayed MMP-20 hydrolysis of the adsorbed amelogenin in a dose-dependent manner (fig. 6) even at the lowest level of fluoride-containing apatite (100 ppm F). This same effect of reduced amelogenin hydrolysis was found when amelogenins were hydrolyzed from fluoride-containing apatites with recombinant KLK-4 (unpublished results).

The levels of fluoride incorporated into the apatite crystals in these in vitro studies are biologically relevant. Although the enamel fluid surrounding the ameloblasts is likely to contain no more than 10 μm (0.19 ppm) fluoride, fluoride is incorporated into the growing crystals in concentrations ranging from 10 ppm near the dentalenamel junction to several thousand ppm at the enamel surface [86]. Fluoride-containing apatite with fluoride concentrations of 100 ppm are found in the inner enamel (300 μm from the surface) of human teeth with minimal (mild) fluorosis [86]. The higher fluoride-containing apatite (approximately 2,000 ppm F) is similar to that found in the midlayer of enamel (150 μm from the surface) of severely fluorosed human teeth. Therefore, these studies indicate that the reduced hydrolysis of amelogenin found in fluorosed maturation stage enamel [1, 52] may be due to the reduction in the rate of hydrolysis of amelogenins bound to fluoride-containing enamel crystals.

These effects of fluoride incorporation on hydrolysis of apatite-bound amelogenins is consistent with the observation that fluoride-induced subsurface hypomineralization can independently occur in the maturation stage only [60, 63, 87]. Mineralization defects in rat incisor maturation stage enamel are characterized by the development of a generalized hypomineralized porous subsurface area along the entire crown enamel [4, 88–91]. This type of defect correlates to the porous white opacities seen clinically.

Potential Effects of Matrix pH on Fluoride-Related Changes in Enamel Formation

Matrix protein removal may also be influenced by fluoride-mediated changes in pH during apatite crystal formation. Formation of apatite results in the formation of a substantial number of protons [$10Ca^{2+} + 6\ HPO^{2-}{}_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 8H^+$] that need to be neutralized. Amelogenins bind as many as 12 protons per molecule [92]. However, if this amelogenin buffering system is either not available, or is saturated, it is conceivable that a

fluoride-induced pH drop could alter the amelogenin tertiary structure and affect its function [93].

Abundant amelogenins generated by secretory ameloblasts may be a potent contributor to controlling pH at the secretory stage, where the pH is maintained at neutral [77, 94]. At the end of the secretory stage, enamel matrix proteinases are activated, and at the transition stage, enamel matrix proteins are rapidly lost. At this stage, the cell junctions between the ameloblasts are open, allowing fluoride to readily move from the serum to the enamel matrix. The presence of increased amounts of fluoride could promote enhanced enamel matrix mineralization with potentially increased amelogenin retention in the presence of a reduced matrix pH.

At the maturation stage, the pH in the enamel matrix changes periodically between acidic (pH 5.8) and neutral as ameloblasts modulate (pH 7.2) [95, 96], and additional pH regulation is required. If we assume that the acidification of the enamel matrix promotes modulation from ruffle-ended to smooth ended ameloblasts during amelogenesis, in dental fluorosis, changes in matrix pH could contribute to a delay in the transition from ruffle-ended to smooth ended ameloblasts, resulting in fewer ameloblast modulations. This delay in ameloblast modulation (which is a characteristic of fluorosed maturation ameloblasts) could possibly contribute to the delay in removal of amelogenins.

Particularly at this final stage of enamel mineralization, Bronckers et al. [94] have hypothesized that fluoride in the enamel matrix may enhance mineralization resulting in localized hypermineralization, requiring the ameloblasts to pump additional bicarbonate into the extracellular enamel matrix. This hypermineralization would deplete the local reservoir or free calcium ions, resulting in a subsequent band of hypomineralized enamel. This hypothesis is supported by a recent study showing an upregulation of mRNA for the pH regulator NBCe1 in fluorosed maturation stage ameloblasts as compared to control maturation ameloblasts [93].

In summary, the mechanisms by which fluoride alters enamel maturation are multi-factorial. We propose a multi-stage model for the formation of fluorosed enamel, as follows:

1. Crystals forming in the secretory stage of enamel have an increased fluoride content and therefore bind more amelogenin.
2. Hydrolysis of amelogenins by proteinases is delayed by altered amelogenin interactions with the fluoride-containing hydroxyapatite crystals.
3. At the transition stage, fluoride is rapidly deposited into the porous enamel matrix between the open cell junctions, resulting in increased formation of fluoride-containing apatite, and a delay in protein hydrolysis secondary altered mineral/ matrix interactions.
4. The net result of these fluoride-related effects in the secretory and transition stages is retention of amelogenins in the maturation stage. This delay in removal of amelogenins increases the relative pH in the maturation stage under ruffle-ended ameloblasts as amelogenins buffer the increased protons resulting from mineral formation.
5. The reduced acidification of the matrix under ruffle-ended ameloblasts further delays modulation to smooth-ended ameloblasts, resulting in fewer bands of modulating ameloblasts.
6. In late maturation, when amelogenins are finally removed (or in mild dental fluorosis with minimal amelogenin retention), fluoride-mediated

hypermineralization may increase the local acidification affecting ameloblast function, such as ion transport activities. Although porous subsurface enamel is the major phenotype of fluorosed enamel, successive layers of hypo-mineralized and hypermineralized enamel are also a characteristic of the fluorosed enamel matrix [4, 90].

It is likely that there are additional effects of fluoride, including other indirect effects on cells at different stages of formation, and that in the course of our and others' studies this model and our understanding of the mechanisms (including more potential direct cellular effects) will be expanded.

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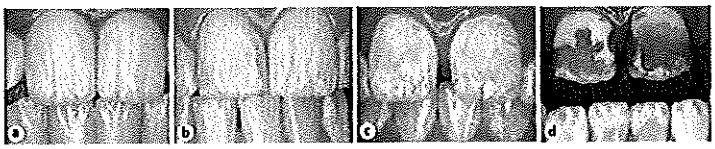

Fig. 1.
Dental fluorosis. **a** Mild with slight accentuation of the perikymata. **b** Moderate, showing a white opaque appearance. **c** Moderate, white opaque enamel with some discoloration and pitting. **d** Severe.

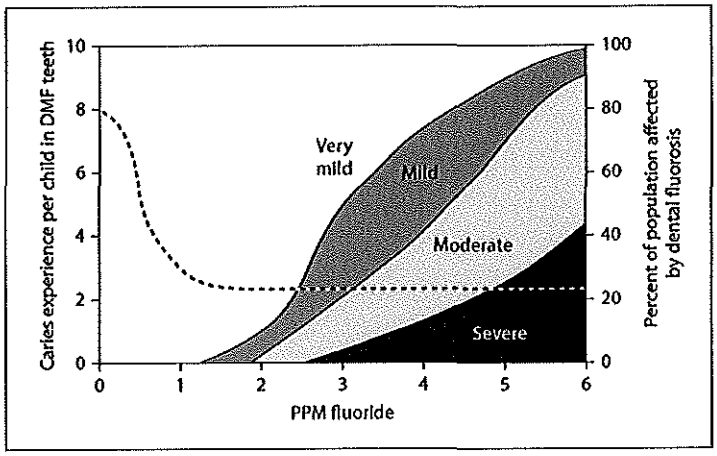

Fig. 2.

Concentrations of fluoride in drinking water are related to caries incidence in children and severity of dental fluorosis. Adapted from a report of the Department of Health and Human Services of US (1991) [24].

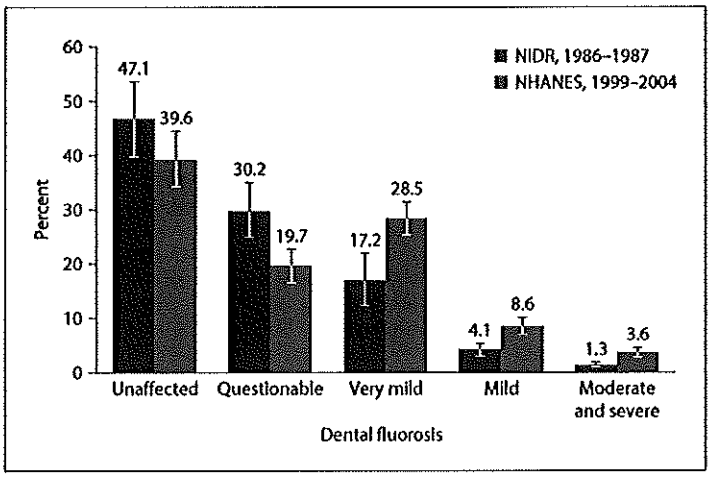

Fig. 3.

Change in dental fluorosis prevalence among children aged 12–15 years participating in 2 national surveys in the USA (1986–1987 and 1999–2004). Dental fluorosis (based on Dean's fluorosis index) is defined as: very mild, mild, moderate or severe. Percentages do not sum to 100 due to rounding. Error bars = 95% CI. Sources: National Health and Nutrition Examination Survey (1999–2004) [27] and National Survey of Oral Health in U.S. School Children (1986–1987) [27].

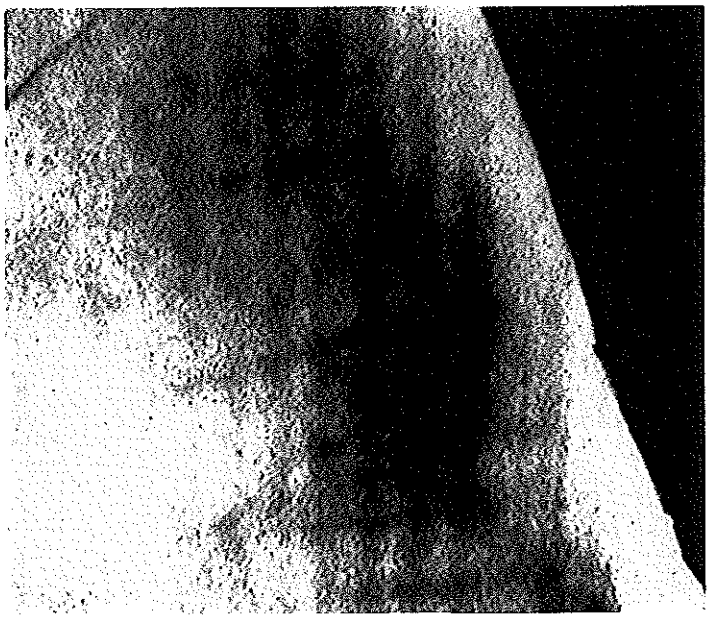

Fig. 4.
Microradiograph of fluorosed enamel from Colorado Springs. Note the radiolucent outer third of the enamel with a well-calcified surface layer. From Newbrun [97], reprinted with permission.

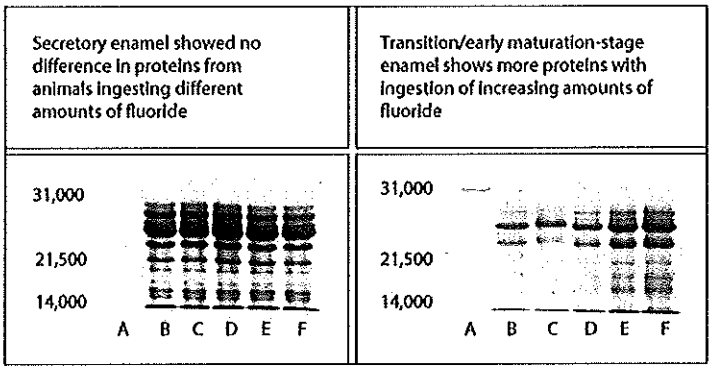

Fig. 5.
SDS PAGE separation of proteins in secretory and maturation stages of enamel matrix of fluoride-treated and untreated rat tooth. A = Standard; B = 0 ppm; C = 10 ppm; D = 25 ppm; E = 50 ppm; F = 100 ppm. From DenBesten [51], reprinted with permission.

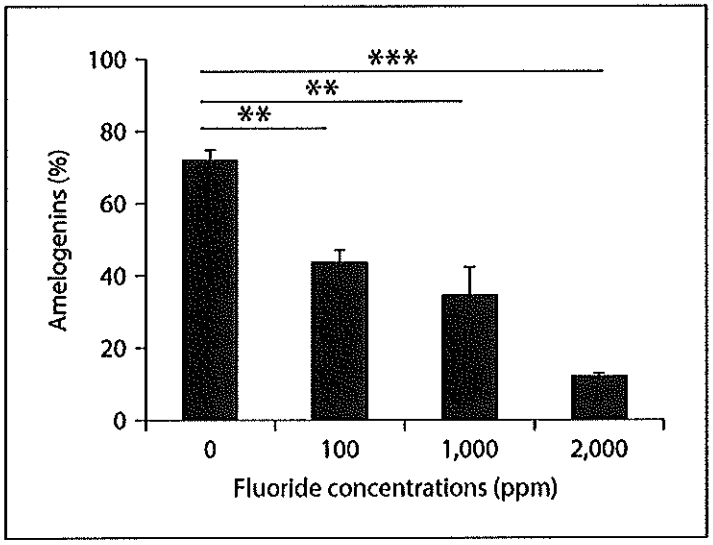

Fig. 6.

Degradation of amelogenin adsorbed on apatite crystals by MMP-20. Amelogenins were pre-bound to carbonated hydroxyapatite crystals containing different amounts of fluoride (X-axis) and then degraded by MMP-20. Y-axis indicates the percentages of amelogenins degraded by MMP-20 from apatite crystals as compared to the amount of amelogenin initially bound. Note the decreased degradation of amelogenin from the apatite crystal surface as the concentration of fluoride in the apatite increases.

Table 1

Fluorosis index of H.T. Dean (1942)

Score	Criteria
Normal (0)	The enamel represents the usual translucent semivitriform type of structure. The surface is smooth, glossy, and usually of a pale creamy white color.
Questionable (0.5)	The enamel discloses slight aberrations from the translucency of normal enamel, ranging from a few white flecks to occasional white spots. This classification is utilized in those instances where a definite diagnosis of the mildest form of fluorosis is not warranted and a classification of 'normal' is not justified.
Very mild (1)	Small opaque, paper white areas scattered irregularly over the tooth but not involving as much as 25% of the tooth surface. Frequently included in this classification are teeth showing no more than about 1–2 mm of white opacity at the tip of the summit of the cusps of the bicuspids or second molars.
Mild (2)	The white opaque areas in the enamel of the teeth are more extensive but do not involve as much as 50% of the tooth.
Moderate (3)	All enamel surfaces of the teeth are affected, and the surfaces subject to attrition show wear. Brown stain is frequently a disfiguring feature.
Severe (4)	Includes teeth formerly classified as 'moderately severe and severe.' All enamel surfaces are affected and hypoplasia is so marked that the general form of the tooth may be affected. The major diagnostic sign of this classification is discrete or confluent pitting. Brown stains are widespread and teeth often present a corroded-like appearance.

As reproduced in National Academy of Sciences [p.169, 16].

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Table 2

Dietary reference intakes for fluoride

Age groups	Reference weight, kg (lb)	Adequate intake, mg/day	Tolerable upper intake, mg/day
Infants 0–6 months	7 (16)	0.01	0.7
Infants 7–12 months	9 (20)	0.5	0.9
Children 1–3 years	13 (29)	0.7	1.3
Children 4–8 years	22(48)	1.0	2.2
Children 9–13 years	40 (88)	2.0	10
Boys 14–18 years	64 (142)	3.0	10
Girls 14–18 years	57 (125)	3.0	10
Males ≥19 years	76 (166)	4.0	10
Females ≥19 years	61 (133)	3.0	10

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Table 3

Enamel fluorosis among persons aged 6–39 years by selected characteristics

	Unaffected		Questionable		Very mild		Mild		Moderate/ Severe	
	%	SE	%	SE	%	SE	%	SE	%	SE
Age group (years)										
6–11	59.81	4.07	11.80	2.50	19.85	2.12	5.83	0.73	2.71	0.59
12–15	51.46	3.51	11.96	1.84	25.33	1.98	7.68	0.93	3.56	0.59
16–19	58.32	3.30	10.21	1.70	20.79	1.78	6.65	0.67	4.03	0.77
20–39	74.86	2.28	8.83	1.23	11.15	1.22	3.34	0.58	1.81	0.39
Sex										
Male	67.65	2.63	9.99	1.45	15.65	1.52	4.58	0.54	2.12	0.39
Female	66.97	2.84	9.83	1.34	15.58	1.36	4.84	0.61	2.78	0.49
Race/ethnicity[1]										
White, non- Hispanic	69.69	3.13	10.43	1.62	14.09	1.56	3.87	0.60	1.92	0.48
Black, non- Hispanic	56.72	3.30	10.40	2.16	21.21	2.16	8.24	0.82	3.43	0.54
Mexican- American	65.25	3.89	8.95	1.29	15.93	2.24	5.05	0.72	4.82[2]	1.81
Poverty Status[3]										
<100% FPL	68.02	3.21	10.67	1.64	14.28	1.73	4.07	0.69	2.97	0.66
100–199% FPL	66.92	2.91	9.11	1.79	16.11	1.46	5.21	0.78	2.65	0.56
≥200% FPL	66.88	2.75	10.73	1.33	15.56	1.56	4.83	0.50	2.00	0.37
Total	67.40	2.65	9.91	1.35	15.55	1.37	4.69	0.49	2.45	0.40

Data from National Health and Nutrition Examination Survey (1999–2002) [27] and calculated using Dean's index. All estimates are adjusted by age (single years) and sex to the USA 2000 standard population, except sex, which is adjusted only by age.

[1] Calculated using 'other race/ethnicity' and 'other Hispanic' in the denominator.

[2] Unreliable estimate: the standard error is 30% the value of the point estimate, or greater.

[3] Percentage of the federal poverty level (FPL), which varies by income and number of persons living in the household.