SHORT STATURE, BONE DEFORMITIES, COGNITIVE IMPAIRMENT, DELAYED DENTAL ERUPTION, AND DENTAL FLUOROSIS AS EXAMPLES OF FLUORIDE-INDUCED DEVELOPMENTAL DISORDERS INVOLVING DISTURBED THYROID HORMONE METABOLISM AND SONIC HEDGEHOG SIGNALLING

ABSTRACT: After considering (i) the ability of fluoride, in amphibians, to inhibit metamorphosis and growth, and to disturb hard tissue ossification; (ii) the control of amphibian metamorphosis by triiodothyronine (T3) and the sonic hedgehog signalling pathway; (iii) human fluoride-induced developmental disorders (FIDD) with short stature, bone deformities, cognitive impairment, delayed dental eruption, and dental fluorosis; (iv) the effects of fluoride on thyroid hormone metabolism and the sonic hedgehog pathway which result in a decrease in the serum T3 and an elevation in the serum rT3; and (v) the evidence linking fluoride exposure, thyroid hormone metabolism, the sonic hedgehog signalling pathway, and the listed human FIDD; it was concluded that these amphibian and human FIDD can be considered to result from disturbed thyroid hormone metabolism and sonic hedgehog signalling.

Keywords: Bone deformities; Cognitive impairment; Delayed dental eruption; Dental fluorosis; Fluoride-induced developmental disorders; Iodine deficiency; Melatonin; Short stature; Sonic hedgehog signalling; Thyroid hormone metabolism.

Fluoride inhibits amphibian metamorphosis and growth, and disturbs hard tissue ossification: As reported by Chen, Chai, Zhao, Wu, and Wang, in the present issue of *Fluoride* on pages 128–142, chronic exposure to 50 mg NaF/L significantly increased the mortality, inhibited metamorphosis, and delayed development, with reductions in the total length and body weight, in *Rana chensinesis* and *Rana nigromaculata* tadpoles.[1] Ossification was reduced in the *Rana nigromaculata* tadpoles and stimulated in the *Rana chensinesis* tadpoles.[1] Their findings are consistent with earlier work on inhibition of metamorphosis,[2-4] and reduction of head-tail length[5] with NaF exposure. Reduced growth in *Rana pipiens* embryos was reported by Cameron at 1 mg NaF/L.[6] In 2013, Zhao, Chai, and Wang found that metamorphosis was strongly inhibited and calcium deposition was retarded in *Bufo gargarizans* tadpoles with 50 mg F/L, while with 5 mg F/L bone mineralization was stimulated.[7] There is thus evidence that fluoride inhibits amphibian metamorphosis and growth, and disturbs hard tissue ossification.

Amphibian metamorphosis is controlled by triiodothyronine (T3) and the sonic hedgehog signalling pathway: Amphibian metamorphosis involves systematic transformations in various tadpole organs and tissues with remodelling, resorption, and *de novo* development through cell proliferation and apoptosis (programmed cell death).[8] Developmental exposure to appropriate levels of thyroid hormones in a timely manner is critical to normal development in vertebrates[9] and reduced or excessive levels can result is severe abnormalities.[9] Gudernatsch found in 1912 that a thyroid extract caused *Rana temporaria* tadpoles to turn into frogs.[10]Thyroxine (T4) was subsequently identified in 1926 and its active metabolite, the thyroid hormone triiodothyronine (T3), in 1952.[11] The selenoprotein deiodinases, D1, D2, and D3, can regulate the activation of T4 and the inactivation of T3 in a tissue specific manner, e.g., during tadpole metamorphosis, T3 can be inactivated in a specific region of the retina by D3 while D2 activates T4 in tail tissues.[12] D2 generates the active form of thyroid hormone T3 via deiodination of T4 while D3 inactivates T3 and, to a lesser extent, prevents T4 from being activated.[13] The Hedgehog cascade, with sonic hedgehog (Shh) decreases D2 activity by promoting D2 ubiquitination.[13] The transition between active and inactive D2 is via ubiquitination and deubiquitination, reactions which are catalyzed by the D2-specific ubiquitin ligase WD repeat and SOCS box containing 1 enzyme (WSB-1) and the von Hippel-Lindau protein-interacting deubiquitination enzymes 1 and 2 (VDU1 and VDU2) respectively.[13] The linking of Hedgehog signalling with thyroid hormone action WSB-1/D2 may play an important homeostatic role in some settings by sustaining a microenvironment of relative hypothyroidism.[13] In the *Xenopus laevis* intestine and limbs during metamorphosis where D2 is expressed and Hedgehog signalling is induced by T3, a negative feedback loop may exist where WSB1-mediated D2 ubiquitination is induced as a result of D2-catalyzed T4 to T3 conversion.[13-15] T3 regulates the expression of both sonic

hedgehog (Shh) and its coreceptors, patched (Ptc) and smoothed (Smo).[15] D3 is responsible in part for the low thyroid hormone levels in the fetus.[9] The overexpression of D3 inhibits metamorphosis in *Xenopus laevis*.[16] D1 is a kinetically inefficient enzyme that activates or inactivates T4 on an equimolar basis and its role in health remains to be clarified.[13] Metamorphosis is blocked in tadpoles by administering drugs that interfere with thyroid function by inhibiting the iodination of thyroglobulin, e.g., methimazole and sodium perchlorate, or by stopping the conversion of T4 to T3, e.g., iopanoic acid.[12] All metamorphic changes are controlled by T3 acting by transcriptional regulation through heterodimers of thyroid hormone receptors (TRs) and 9-*cis* retinoic acid receptors (RARs).[8,17,18] Amphibian metamorphosis is tightly regulated by the T3 and sonic hedgehog (Shh) signalling pathways.[19]

Fluoride-induced developmental disorders (FIDD): The inhibition of metamorphosis, the reduced head-tail length, and the disturbed hard tissue ossification in tadpoles with exposure to fluoride ions can be described as examples of fluoride-induced developmental disorders (FIDD). FIDD may be considered to be disorders in which fluoride ions have disrupted, with arrest, reduction, delay, or acceleration, the normal development, growth, or maturation of an organism, at some stage between the commencement of the life of the organism and its becoming an adult.

FIDD in humans: Reports of FIDD from humans with reduced, delayed, or accelerated development and growth include (i) *earlier onset of female sexual maturity*: e.g., girls in fluoridated Newburgh, New York, USA, had an average age for starting to menstruate (menarche) of 12 yr compared to 12 yr 5 months for those in the nonfluoridated control city of Kingston.[20] (ii) *short stature*: e.g., adolescents, aged 15–20 yr, using water with 5.3 mg F/L in Villa Ahumada, Mexico, were 5.7 cm shorter in height than those using water with 0.3 mg F/L;[21,22] (iii) *bone deformities*: e.g., children and adolescents aged less than 20 yr in Tilaipani, Mandla District of Central India, using drinking water with 9.22–10.83 mg F/L had a prevalence of genu valgum (knock knee) of 51.2% and similarly aged children and adolescents in nearby Hirapur, using drinking water with <1–13.5 mg F/L had a prevalence of genu valgum of 6.5%;[23] (iv) *cognitive impairment*: e.g., children, aged 8–13 yr, in Wamiao, PR China, using drinking water with 2.47±0.79 mg F/L had a lower mean Intelligence Quotient (IQ), 92.02±13.00, than similarly aged children in Xinhuai, using drinking water with 0.36±0.15 mg F/L, whose mean IQ was 100.41±13.21;[24-26] (v) *delayed dental eruption*: e.g., children, aged 12 yr, in Colorado Springs, using drinking water with 2.6 mg F/L, had on average more than two fewer erupted permanent teeth than children in low fluoride cities;[27] when tablets containing 1 mg of the fluoride ion were given to pregnant women and their children through to when the children were aged 8 yr, many children showed a marked delay in the eruption of the deciduous teeth, in many cases by as much as a year from the accepted average eruption dates and when the delayed teeth did erupt, they did so as a group, all within a period of a few days;[28] and (vi) *dental fluorosis*: e.g., a prevalence of dental fluorosis of 81.7% was found in 800 individuals, aged >5–<60 yr, living in 33 rural communities in Chihuahau, Mexico, with a mean drinking water concentration of 3.74 mg F/L, range 0.7–8.6 mg F/L;[29]

Aetiological factors in FIDD: Whether or not one of the FIDD occurs in a particular situation depends not only on the level and duration of the fluoride exposure but also on the presence of predisposing and protective factors including: (i) species differences;[1] (ii) genetic differences;[30] (iii) water hardness, water calcium and magnesium levels, and dietary intake of cations;[31,32] (iv) dietary intake of vitamins, antioxidants, and selenium[33-35] although at higher levels selenium is synergistic with fluoride and arsenic in causing toxicity;[36] (v) dietary intake of iodine and dietary goitrogens;[37-39] (vi) exposure to other toxic chemicals and drugs;[40-43] and (vii) altitude.[44-46]

Pathophysiology of FIDD: Different pathophysiological mechanisms may be involved in the various FIDD. An *earlier onset of female sexual maturity* may be partly the result of fluoride interfering with the synthesis, from tryptophan in the pineal gland, of melatonin, which may have a role in the timing of the onset of puberty.[47] The human pineal gland has a rich blood supply, accumulates fluoride, and contains the highest concentration of fluoride in the body.[47,48] Fluoride is associated with depressed pineal melatonin synthesis by prepubertal gerbils and an accelerated onset of sexual maturation in the female gerbil.[47] *Short stature, bone deformities, cognitive impairment, delayed dental eruption,* and *dental fluorosis* may all occur in hypothyroidism and it is appropriate to consider whether their occurrence in FIDD may be the result of fluoride interfering with thyroid hormone metabolism and the sonic hedgehog pathway during development.

Effects of fluoride on thyroid hormone metabolism and the sonic hedgehog signalling pathway: Zhao, Chai, and Wang found in *Bufo gargarizans* tadpoles that 50 mg

F/L could damage follicular cells in the thyroid gland and induce a sharp reduction in thyroid hormone, probably through the up-regulation of D3 mRNA expression, and that these influences on the thyroid system may delay metamorphosis as well as ossification in bone tissues by inhibiting calcium deposition.[7] In a study of 250 7–14-yr-old-children in an area with a high drinking water fluoride level (0.88 mg F/L) and a low drinking water iodine level (5.21 mg I/L), 256 7–14-yr-old-children in an area with a low drinking water fluoride level (0.34 mg F/L) and a low drinking water iodine level (0.96 mg I/L), and 243 7–14-yr-old-children in a area with iodine supplementation in salt or oil, the balance of active T3 and inactive rT3 in the serum was seen to reflect the thyroid hormone economy.[32] In the high fluoride and low iodine areas, the rT3 was 58 ng/dL (normal 21 ng/dL) and the ratio of rT3/T3 was significantly low (2.91).[38] In the low fluoride and low iodine area, the rT3 value was 32 ng/dL and the ratio of rT3/T3 was 5.8.[38] Five patterns of thyroid hormone derangements were found in 7–18-yr-old-children living in fluoride endemic, non-iodine deficient, areas in India: (i) high TSH with normal T4 and T3 (46.9%); (ii) low T3 with normal TSH and T4 (32.7%); (iii) high TSH and T3 with normal T4 (14.3%); (iv) high TSH and low T4 with normal T3 (4.1%); and (v) high TSH and low T3 with normal T4 (2.0%).[49] A decreased T3 level (132.9±26.8 ng/dL) was present in children, aged 15–20 yr, in Samalayuca, Mexico, with 1 mg F/L in their drinking water compared to the level (149.7±20.9 ng/dL) in children with a drinking water fluoride concentration of 0.3 mg F/L.[21,22] In a study of 279 adults, aged 22–47 yr, from non-fluorotic (140) and endemic fluorosis (139) areas with water fluoride levels of 0.65–1.00 mg F/L and 1.01–16.00 mg F/L, respectively, increased exposure to fluoride was associated with a low level of T3, a high rT3, a slight increase in the TSH, and a normal or low T4.[50] In 50 adults, aged 25–35 yr, using water with 0.76 –16.00 mg F/L, with greater fluoride exposure the serum TSH and rT3 increased and decreases occurred in the serum T3, T4, free T3 (FT3), free T4 (FT4), D1, and D2.[51] A direct correlation was present between the serum fluoride and the reverse T3 (rT3).[51] D2 generates the active form of thyroid hormone T3 via deiodination of T4 while D3 inactivates T3 and, to a lesser extent, prevents T4 from being activated.[17] The presence of a decreased serum T3 with an elevation in the serum rT3, in the presence of normal or optimal levels of urinary iodine, has been seen to be diagnostic of chronic fluoride exposure.[50] Fluoride has variable effects on Shh expression with inhibition described in Shh secretory ameloblasts[52-53] and increased expression occurring in hepatocytes[54-55] and primary chondrocytes.[56] Fluoride can interfere with thyroid hormone metabolism with the effects on the deiodinases D1, D2, and D3 and the sonic hedgehog signalling pathway being central.

Mechanisms by which fluoride is toxic: Fluoride toxicity may arise by a variety of mechanisms. Fluoride, in the form of an aluminofluoride complex (AlF_x), is a phosphate group analogue which is able to mimic thyroid stimulating hormone (TSH) by switching on its associated G protein and it is suggested that the consequent overproduction of the second messenger cAMP leads to a feedback mechanism resulting in a desensitization of the TSH receptor and ultimately to a reduced activity of the thyroid gland.[57,58] The complexes formed by fluoride with metals such as aluminum and beryllium which mimic phosphate, such as AlF_4^- and BeF_3^-•H2O, have either positive or negative effects on a variety of enzymes and regulatory phosphatases.[59-61] In whole-cell systems, the intracellular effects of extracellular Al^{3+} and fluoride are often biphasic being stimulatory in low doses and inhibitory at high doses.[60] At low doses, biological systems display an overcompensation response which results in the apparent low-dose stimulation toxicity.[60] At higher doses with greater toxicity, the system often displays a more limited capacity for a compensatory response which is usually insufficient to return to the control levels.[60] Fluoride may also stimulate heterotrimeric G proteins in an Al^{3+}- or Al^{4+}-independent manner,[62] possibly by acting as a phosphatase inhibitor.[63] Hydrogen fluoride, HF, is a weak acid and can act as a transmembrane proton conductor and de-energize the cell membrane was discharging ΔpH.[59] Fluoride inhibits enzymes containing a metal, such as cytochrome oxidase containing Fe, enzymes that need a metal ion for activity, such as enolase which requires Mg^{2+}, and enzymes not containing or needing a metal ion for activity such as acetylcholinesterase where toxicity may follow the breaking up of existing hydrogen bonds and the formation of new ones.[64] Fluoride may stabilize activated receptors[65] and threaten microtubule stability.[66]

*The aetiological role of fluoride interference with thyroid hormone metabolism and the sonic hedgehog signalling pathway in the FIDD involving impaired growth and disturbed hard tissue ossification***:** The inhibition of amphibian metamorphosis, together with impaired growth and disturbed hard tissue ossification, by fluoride is a result of fluoride disturbing thyroid hormone metabolism and the sonic hedgehog

signalling pathway. It is appropriate to consider whether the mammalian FIDD with impaired growth and disturbed hard tissue ossification (short stature, bone deformities, cognitive impairment, delayed dental eruption, and dental fluorosis) have a similar aetiology.

Fluoride exposure, thyroid hormone metabolism, the sonic hedgehog signalling pathway, short stature, bone deformities, cognitive impairment, delayed dental eruption, and dental fluorosis: A significant inverse relationship was found between urine fluoride levels and stature in children in Villa Ahumada, Mexico, with 5.3 mg F/L in their drinking water.[21,22] Short stature, retarded bone age with delayed fusion of the epiphyses and diaphyses of the long bones, cognitive impairment, delayed dental eruption, and enamel hypoplasia with increased porosity, the same as the lesions seen in dental fluorosis, can all occur in congenital hypothyroidism.[67-70] Angular limb deformities in foals have been associated with hypoplasia and incomplete ossification of the carpal and tarsal bones.[71] Foals with congenital hyperplastic goitre had retarded ossification of the cuboidal bones, especially the third and central tarsal bones.[71] Foals who were thyroidectomized when one-day-old had retarded ossification to a lesser degree.[71]

Thyroid hormone and growth hormone have a permissive action and both have to be present for normal growth to occur.[67] The fluoride effects on thyroid function are more severe in the presence of iodine deficiency. In the village of Ban Mae Toen, Thailand, adjacent to a polluted artificial lake water source, containing 4 mg F/L and less than 0.1 mg I/L, formed as a result of fluoride mining 40 years ago and still being used in 2007 in the dry season, 11% had goitre (20% of the women and 3% of the men) compared to 0% in two nearby control villages, 24% had lower limb deformities compared to 5% in the control villages, and 63% had discoloured teeth, consistent with dental fluorosis, which was significantly more than in the control villages, $p<0.01$.[72,73] Brain damage, deaf-mutism, and mental disability were reported in the children of pregnant women who drank the contaminated water.[73] A further example of impaired growth and development affecting the teeth and bones is a 20-yr-old patient with congenital hypothyroidism, short stature (114 cm), subnormal weight for her age (20 kg), and a mixed dentition with 11 retained deciduous teeth and 15 unerupted permanent teeth.[67] Radiographs showed delayed closure of the coronal, sagittal, and lamboid sutures, delayed fusion of the epiphysis and diaphysis of the phalanges, metacarpals, radius, and ulna, and non-ossification of the sesamoid bone and the hook of the hamate.[67]

Interference with thyroid hormone metabolism during development may lead to various failures of maturation which may not be fully reversed by later treatment with thyroxine. The failure of appropriate limb ossification may lead to irreversible limb deformities.[23] Although administering thyroid hormone up to the age of 19 yr may result in exfoliation of the primary dentition and eruption of the secondary dentition, the lack of proper growth on the mandible and failure of the normal resorption of the internal aspect of the ramus may lead to impaction of the mandibular second molars.[68] The failure to properly remove amelogenic proteins and organic matrix components may lead to dental fluorosis with increased enamel porosity and impaired enamel crystal growth.[74-76] The degree of fluorosis is greatest among those teeth formed later during childhood.[77] Fluoride may replace hydroxyl ions in calcium hydroxyapatite in hard tissue to form calcium fluoroapatite and then be toxic to the respective cells of the hard tissues leading to dental fluorosis, osteomegaly and osteopenia.[78,79] Since the the alveolar bone has a higher metabolic rate, than skeletal bone tissue at other sites, the recession of the alveolar bone and gingival tissue is an important sign of chronic fluoride toxicity.[79,80]

Lower limb deformities are less common and may result from the presence of particularly high fluoride levels or, possibly, fluoride toxicity being exacerbated by iodine deficiency. Xiang et al.[24] did not report any lower limb deformities in their study which found a significantly reduced IQ in children in Wamiao, PR China, with a drinking water fluoride level of 2.47±0.79 mg F/L while McGlashan et al.[72] found 24% of the Thai residents adjacent to lake water with 4 mg F/L, had lower limb deformities, compared to 5% in two control villages. However, the children in Wamiao had adequate urinary iodine levels, 280.70±87.16 µg/L[24] while the water sources in the study and control villages in Thailand had iodine levels below the detection limit of 100 µg/L.[72] A median urinary iodine level of 50 µg/L or less has been proposed for classifying iodine deficiency and a level of 100–199 µg/L is an adequate intake for school age children aged 6 or more years.[81] Iodine levels were not reported on in the endemic genu valgum outbreak in Mandla District of Central India but the condition only appeared there after deep bore wells began to be used as a water source and the most severe cases were in Hirapur where the only hand pump had a water fluoride level of 13.5 mg F/L.[23] Similarly, the occurrence of endemic genu valgum associated with fluorosis in Andra Pradesh,

India, in 1973 followed fluoride contamination of surface water by underground water due to the construction of a dam in a nearby area.[82,83] The differential diagnosis of genu varu, genu valgum, and other bone deformities includes rickets due to vitamin D deficiency.[84]

Dental fluorosis, with increased enamel porosity and impaired enamel crystal growth, results from a failure to properly remove amelogenic proteins and organic matrix components[74-73] and is usually discussed without reference to thyroid hormone metabolism.[74,85-88] However, extracellular remodelling plays an important role during tissue remodelling in amphibian metamorphosis and extracellular matrix degrading metalloproteinases are expressed in response to thyroid hormone.[17] Sonic hedgehog (Shh) regulates the growth and morphogenesis of the tooth and when Shh is absent the polarity and organization of the ameloblast and odontoblast layers is disrupted.[89] Thyroid hormone regulates the expression of the sonic hedgehog signalling pathway in the embryonic and mammalian brain.[15] This suggests that thyroid hormone metabolism may be relevant in the development of dental fluorosis in agreement with Schuld who found in 2005 that understanding thyroid hormone metabolism was essential in understanding fluoride toxicity including dental and skeletal fluorosis and the effects on IQ.[90] Although dental fluorosis is usually considered mainly with respect to tooth appearance and function, it is also important as a marker for fluoride-induced cognitive impairment.[91]

Conclusion: Thus, the syndromes, after fluoride exposure, in amphibians, of inhibited metamorphosis and growth and of disturbed hard tissue ossification, and, in humans, of short stature, bone deformities, cognitive impairment, delayed dental eruption, and dental fluorosis, can be considered to be examples of FIDD in which the pathophysiology involves disturbed thyroid hormone metabolism and sonic hedgehog signalling. Other FIDD, such as the earlier onset of female sexual maturity, may involve different pathophysiological mechanisms such as fluoride reducing the pineal gland synthesis of melatonin, which may have a role in the timing of the onset of puberty, although melatonin levels may also be lowered by induced hypothyroidism.[92]

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