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ABSTRACT

Concurrent with the decline in dental caries has been an increase in the prevalence of dental fluorosis, a side-effect of exposure to greater than optimal levels of fluoride during amelogenesis. The mechanisms that underlie the pathogenesis of dental fluorosis are not known. We hypothesize that genetic determinants influence an individual's susceptibility or resistance to develop dental fluorosis. We tested this hypothesis using a mouse model system (continuous eruption of the incisors) where genotype, age, gender, food, housing, and drinking water fluoride level can be rigorously controlled. Examination of 12 inbred strains of mice showed differences in dental fluorosis susceptibility/resistance. The A/J mouse strain is highly susceptible, with a rapid onset and severe development of dental fluorosis compared with that in the other strains tested, whereas the 129P3/J mouse strain is least affected, with minimal dental fluorosis. These observations support the contribution of a genetic component in the pathogenesis of dental fluorosis.

KEY WORDS: dental fluorosis, inbred mouse strains, quantitative light-induced fluorescence.

Dental Fluorosis: Variability among Different Inbred Mouse Strains

INTRODUCTION

According to the Centers for Disease Control (CDC), fluoridation of community drinking water for the prevention of dental caries is considered to be one of the ten most important public health achievements of the 20th century (1999). Concurrent with the decline in dental caries has been an increase in the prevalence of dental fluorosis, a side-effect of systemic exposure to greater than optimal levels of fluoride during amelogenesis. The prevalence of dental fluorosis in recent years ranges from 7.7% to 80.9% of the population in communities with fluoridated water and from 2.9% to 42% in communities with nonfluoridated water (Clark, 1994; Mascarenhas, 2000; Pendrys, 2000). Although the increase in prevalence has occurred primarily in very mild and mild forms of dental fluorosis, there is evidence that the prevalence of moderate and severe fluorosis is increasing as well (Clark, 1994). In addition to those major risk factors previously identified (use of fluoridated drinking water, fluoride supplements, fluoride toothpaste, and infant formulas) (Mascarenhas, 2000), other factors, including critical periods of fluoride exposure during tooth development and individual variation, are important as well.

While it is well-accepted that fluoride interacts with mineralized tissues and, at elevated concentrations, disturbs the mineralization process (Aoba and Fejerskov, 2002), the molecular mechanisms that underlie the pathogenesis of dental fluorosis are not known. We hypothesize that genotype can influence susceptibility or resistance to develop dental fluorosis.

MATERIALS & METHODS

Mice

From The Jackson Laboratory (Bar Harbor, ME), we obtained weanling (three-week-old) male mice representing the following inbred strains: 129P3/J, A/J, BALB/cJ, C3H/HeJ, C57BL/6J, C57BL/10J, CBA/J, DBA1/J, DBA2/J, FVB/NJ, SJL/J, and SWR/J. The selection of these mouse strains was based upon their phenotypic and genetic differences (the degree of unrelatedness from each other) as well as their general availability as normal laboratory strains. All mice were housed within the Indiana University School of Dentistry Bioresearch Facility, a fully AAALAC-accredited unit. Mice were contained in boxed caging and allowed food and water *ad libitum*. This study was fully approved by the Indiana University School of Dentistry IACUC. Mice were fed a laboratory rodent diet #5001 (PMI® Nutrition International, Richmond, IN, USA). Fluoride determinations of different batches of rodent

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diet fed to the mice were performed by a modified diffusion method (Rojas-Sanchez *et al.*, 1999). The fluoride concentration in the diet was found to range from 5.0 $\mu\text{g/gm}$ to 8.0 $\mu\text{g/gm}$ (mean $7.2 \pm 0.8 \mu\text{g/gm}$).

Fluoride Treatment

Three levels of fluoride treatment were delivered *ad libitum* in the drinking water (0, 25, and 50 ppm F) as NaF. The de-ionized and prepared water samples were periodically analyzed by ion-specific electrode (ISE) for fluoride concentration. The de-ionized water tested from multiple sites within the IUSD Bioresearch Facility showed $[\text{F}] = 0.02 \pm 0.01$ ppm. Each batch of prepared 25 ppm and 50 ppm water samples used during the study was analyzed, and $[\text{F}] = 24.49 \pm 0.27$ ppm and 49.68 ± 0.51 ppm, respectively.

Three treatment groups consisted of 72 mice each (6 each from the 12 strains studied). Group 1 received distilled water, group 2 received distilled water supplemented with 25 ppm fluoride, and group 3 received distilled water supplemented with 50 ppm fluoride.

Oral Examinations

All mice were uniquely identified, and the two examiners (ETE and MAKM) were blinded to the mouse strains and to the treatment groups. All mice were monitored daily for any changes in health status. Once a week, each mouse was given a complete oral examination where incisor tooth color was assessed along the entire labial surface of the enamel by means of a shade guide (Vita Shade Guide, Vident, Brea, CA, USA). The shade guide was analyzed according to the CMYK (cyan, magenta, yellow, and black) color model in the Photoshop 5.5 software, permitting the selective grouping of shades based upon yellow content. This reduced the observations to an ordinal scale (1-3) where 1 = predominantly shades of white, 2 = yellow/tan, and 3 = shades of deep yellow to yellow-orange. Opacity (adjudged when a periodontal probe was passed behind each incisor), length (in mm with the use of a periodontal probe), enamel wear pattern, and overall development of the upper and lower incisors were also independently judged. The determination of dental fluorosis was made clinically over the entire upper and lower incisor tooth surfaces according to a modified TF index (Thylstrup and Fejerskov, 1978). At day 60 into the treatment, mice were killed, weighed, and examined, and selected mineralized tissues were removed.

Quantitative Light-induced Fluorescence (QLF)

One pair of mandibular central incisors from each animal was dissected, rinsed briefly in 10% formalin, and placed in a labeled microfuge tube. A QLF camera handpiece was attached to a vertical camera stand at a fixed distance of 5 cm above a black cardboard plate. The lower incisors were laid labial side up, flat on the cardboard, and stabilized with wax if needed. An image was taken of the teeth with the QLF handpiece connected to a control-box (Inspektor Research Systems BV, Amsterdam, Netherlands) and analyzed by QLF software, version 2.00d. The software captured an image of the teeth using 10 frames to create an original image in a bitmap format. The areas of the teeth that demonstrated fluorosis by clinical examination showed up as bright areas on the QLF images, indicating an increase in fluorescence. The images were further processed by Photoshop 5.5 software, with the original images inverted such that white lesions appeared dark. The inverted images were then analyzed by the algorithm in the QLF software, version 2.00d, for quantification of the average increase in fluorescence and the size of the affected area of the teeth.

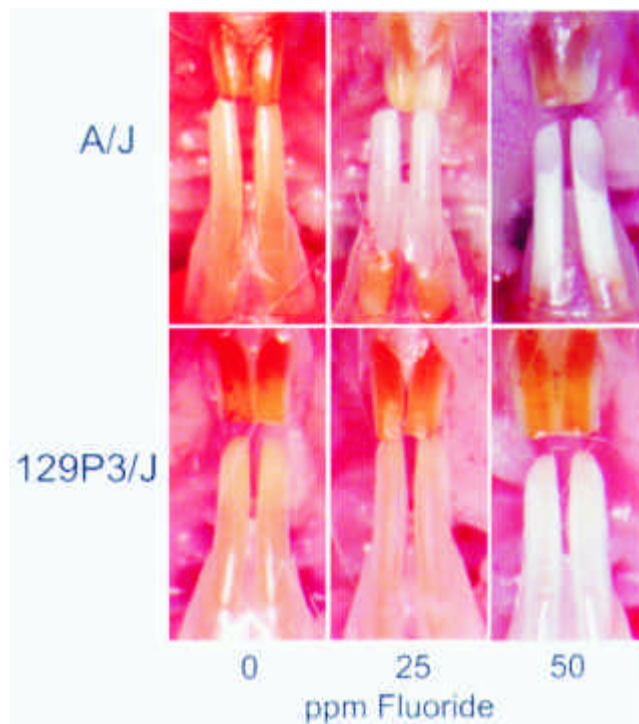


Figure 1. Robust dental fluorosis seen in the A/J strain. Shown are representative A/J and 129P3/J mice after 60 days of treatment (0 ppm, 25 ppm, or 50 ppm fluoride as NaF in the drinking water). Upper and lower incisors of the A/J mice show dramatic color and character changes in response to fluoride. The diffuse yellow-orange color present in the teeth at 0 ppm is typical of normal tooth color development in A/J and other mice. In the 0 ppm treatment group, the lower incisors are translucent as judged by the passing of a periodontal probe behind the teeth. That translucency is not detectable at 25 ppm and 50 ppm $[\text{F}]$. Also in the A/J mice, the labial surfaces of the lower incisors became rough and pitted. At sites where there is a transition (distal aspects), ledges can be detected with probing. The 129P3/J strain is resistant to the development of dental fluorosis. Clinical changes in tooth color and opacity are seen only at the 50-ppm treatment level and are greatly reduced compared with those in A/J mice.

Fluoride Analyses

De-ionized and fluoridated water samples were analyzed directly for fluoride concentration by means of a combination fluoride-specific electrode (Orion #96-909-00) and an Accumet 950 pH/ion meter (Fisher Scientific, Itasca, IL, USA). Analyses of mouse mineralized tissues and mouse chow were performed in duplicate by a modification (Rojas-Sanchez *et al.*, 1999) of the hexamethyldisiloxane (HMDS; Sigma Chemical Co., St. Louis, MO, USA) micro-diffusion method of Taves (1968).

Statistical Analyses

Data were analyzed by descriptive statistics, analysis of variance (one-way ANOVA), Student's *t* test, or Pearson's Correlation where appropriate. Values of $p \leq 0.05$ were considered significant.

RESULTS

Weekly oral examinations of study mice showed the onset of fluoride-related color changes in the incisors (dental fluorosis) to be gradual in some strains (*i.e.*, BALB/cJ and FVB/NJ). Other strains (*i.e.*, A/J, SJL/J, C57BL/6J, and C3H/HeJ) developed dental fluorosis more quickly. A few mouse strains, like 129P3/J and CBA/J, developed minimal

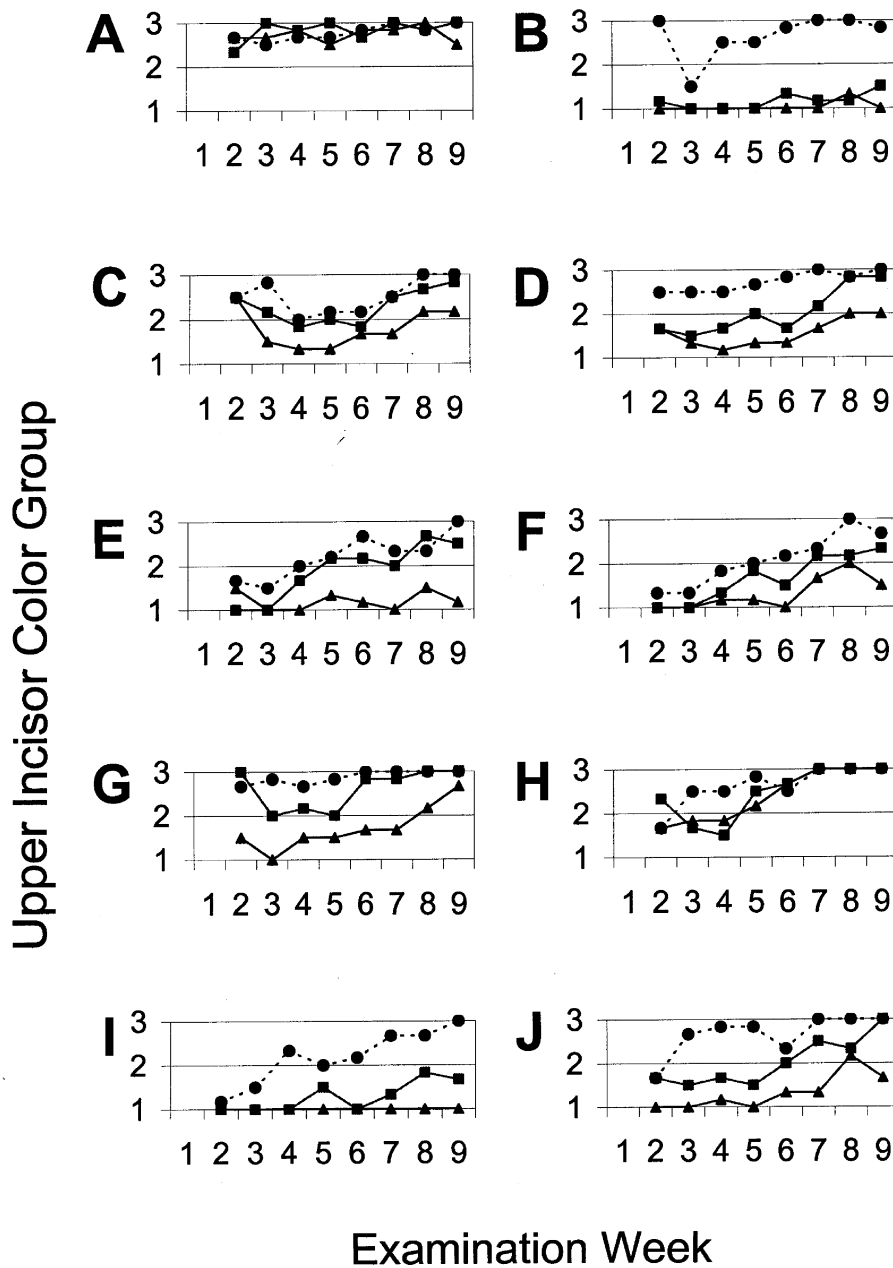


Figure 2. Upper incisor color charting. The Vita shade guide color code converted to color groups 1 (whiter), 2 (yellow/tan), or 3 (deep yellow/orange), and the average value for the 6 mice from each strain per treatment group plotted over time in wks (Panels: **A** = 129P3/J; **B** = A/J; **C** = BALB/cJ; **D** = C3H/HeJ; **E** = C57BL/6J; **F** = C57BL/10J; **G** = CBA/J; **H** = FVB/NJ; **I** = SJL/J; and **J** = SWR/J). These data show that mouse strains respond differently to fluoride treatment and that tooth color changes are dose-responsive. 0 ppm [F] = solid circles with dashed lines, 25 ppm = solid squares, and 50 ppm = solid triangles.

dental fluorosis. While all strains developed various severities of dental fluorosis at 50 ppm [F], some strains were also responsive at the 25-ppm-[F] level. When dental fluorosis developed, the upper incisors and lower incisors appeared white, chalky opaque, and thin. Striations and erosions of the enamel were particularly prominent on the lower incisors. Quite robust fluorosis was seen in the A/J strain (Fig. 1). Dental fluorosis in the A/J strain developed very early, within a couple of weeks, and was manifested at both 25- and 50-ppm-[F]-treated groups. In contrast, the

129P3/J strain (Fig. 1) was the most resistant strain tested, showing minimal dental fluorosis only at the 50-ppm-[F] level. The maxillary and mandibular incisors shown in the 0-ppm-[F] panel illustrate normal tooth development at 13 wks of age for both A/J and 129P3/J mice.

Upper Incisor Color as an Index of Fluorosis

As the incisors erupted, natural color development was modified during the 60-day course of fluoride treatment. Diminished tooth color, as an indicator of dental fluorosis development, was dose-responsive to fluoride exposure (Fig. 2). When the color scale was reduced to an ordinal scale of 1-3, the examiners' observations were highly mutually correlative. Inter-examiner variation for upper incisor color determination was measured by Pearson's Correlation, two-tailed, for each strain, $r = 0.649$ to 0.832 (mean = 0.740 ± 0.06) and significant at $p < 0.01$. Monitoring these tooth color changes added dimension to the clinical characterization of dental fluorosis in these strains of mice.

Longitudinal study of upper incisor color permitted mice to be grouped into resistant (129P3/J, FVB/NJ, CBA/J, and DBA/1J), intermediate (SWR/J, BALB/cJ, C57BL/10J, and DBA/2J), and sensitive (A/J, SJL/J/ C3H/HeJ, and C57BL/6J) strains.

Quantitative Light-induced Fluorescence (QLF) Analysis as a Quantitative Measure of Fluorosis

Mouse mandibular incisors were subjected to QLF analysis. The areas of the teeth that were affected by fluorosis as judged by clinical examination showed up as bright areas on the QLF images, indicating an increase in fluorescence. The

amount and area of the fluorescent signal were sufficient to allow us to discriminate among groups of animals with different severities of dental fluorosis (Table 1). There was strong correlation between QLF of the lower incisors and that in the upper incisor exams (Pearson's Correlation, two-tailed, $r = 0.766$, $p = 0.004$), and there was also modest correlation between QLF and the modified TFI scale used in the clinical determination of dental fluorosis (Pearson's Correlation, two-tailed, $r = 0.558$, $p < 0.001$). We show that QLF analysis provides a quantitative measurement of dental fluorosis. For the

resistant 129P3/J strain, there was no significant difference between Delta Q at 0 ppm [F] compared with 50 ppm [F] ($p = 0.413$), whereas, in the susceptible A/J strain, the Delta Q at 0 ppm [F] compared with 50 ppm [F] was significant ($p = 0.006$). Furthermore, the difference between the most sensitive strain (A/J) and the most resistant strain (129P3/J) is large. At 50 ppm, A/J vs. 129P3/J $p = 0.005$ (Student's t test, two-tailed, paired).

Fluoride Accumulation in Femurs and Incisors as Indicators of Exposure

We analyzed body fluoride burden by determining the quantity of fluoride in mineralized tissues (femurs and mandibular incisors). Femur fluoride ($\mu\text{g F/mg}$ of ashed bone) was determined for all mice across all 12 strains studied. There was significant correlation between fluoride concentration in water and fluoride concentration in the femur, $r = 0.869$, $p < 0.00001$ (Pearson's Correlation, two-tailed). When we compared the femur [F] for each strain at each fluoride treatment dose, there were no significant differences at 0 ppm [F] ($p = 0.719$) and at 50 ppm [F] ($p = 0.06$). At 25 ppm [F] ($p < 0.01$), differences were noted. Overall, all mice accumulated similar amounts of fluoride in their long bone, regardless of whether they exhibited mild to severe dental fluorosis. This was true for the most susceptible (A/J) and the most resistant (129P3/J) strains (Table 2). At 50 ppm [F], there were no significant differences for femur [F] between the 129P3/J and A/J strains ($p = 0.559$). The total [F] in the entire incisor (dentin and enamel) from the gum line to the tip was measured in the dental-fluorosis-susceptible (A/J) and -resistant (129P3/J) strains (Table 2). The accumulated fluoride in the incisor correlated well with fluoride exposure (Pearson's Correlation, two-tailed, of drinking water [F] vs. incisor [F], $r = 0.799$, $p < 0.00001$), and no significant differences were seen between the fluorosis-susceptible and -resistant strains and at either [F] concentration, $p = 0.457$.

DISCUSSION

Many studies have identified the rodent as a useful model for determining the effects of fluoride on dental development (reviewed by Richards, 1990). The mouse remains an important model for aiding our understanding of the pathogenesis of dental fluorosis for several reasons. First, the continual growth of mandibular and maxillary incisors in mice permits the effects of fluoride on new enamel formation to be monitored (Ness, 1965). Second, each inbred mouse strain represents a genetically homogenous group that often differs quite remarkably from mice in another inbred strain (Atchley and Fitch, 1991). Finally, inbred mouse strains have been used for genetic studies because of the isogenicity within a strain and the genetic heterogeneity

Table 1. Quantitative Light-induced Fluorescence (QLF) Analysis of Mandibular Incisors in Dental-fluorosis-resistant (129P3/J) and Dental-fluorosis-susceptible (A/J) Mouse Strains

Strain	[F] ppm ^a	Delta Q ^b	Student's t test ^c 0 ppm [F] vs. 50 ppm [F]
129P3/J	0	0.2 ± 0.1 (N = 6)	$p = 0.413$
	50	0.4 ± 0.9 (N = 6)	
A/J	0	0.1 ± 0.2 (N = 6)	$p = 0.006$
	50	35.5 ± 12.8 (N = 6)	

^a Fluoride concentration present in water given *ad libitum* throughout the study period (60 days).

^b Delta Q = [Delta F (average loss of fluorescence) × area (mm²)] (mean ± SD), QLF performed in triplicate on six pairs of mandibular incisors *per* strain and treatment group.

^c Student's t test, two-tailed, paired, where $p \leq 0.05$ is considered significant. Comparison of A/J vs. 129P3/J at 50 ppm [F], $p = 0.005$.

Table 2. Fluoride Determinations in Femurs and Mandibular Incisors of 129P3/J and A/J Mice

Mouse Strain	[F] ^a	Mean Incisor [F] ^b	Mean Femur [F] ^b
129P3/J	0	449.9 ± 277.9 (N = 6)	335.6 ± 92.3 (N = 6)
	25	1106.1 ± 510.9 (N = 6)	2962.2 ± 734.8 (N = 6)
	50	2047.5 ± 332.9 (N = 6)	3413.5 ± 221.6 (N = 6)
A/J	0	169.4 ± 69.0 (N = 6)	293.6 ± 71.9 (N = 6)
	25	1157.8 ± 585.8 (N = 6)	2221.5 ± 289.8 (N = 6)
	50	2758.2 ± 1260.5 (N = 6)	3295.0 ± 628.5 (N = 6)

^a [F] as ppm present in drinking water given *ad libitum* as NaF throughout the study period (60 days).

^b [F] as $\mu\text{g/g}$ of ashed incisor or femur. One-way ANOVA was used to compare each mouse strain with another for femur [F] or incisor [F] concentrations at each treatment group. At 50 ppm [F], there were no significant differences for femur [F] between the 129P3/J and A/J strains ($p = 0.559$) or for incisor [F] between the 129P3/J and A/J strains ($p = 0.457$). Fluoride exposure correlated with [F] in mineralized tissues, Pearson's Correlation, drinking water [F] vs. femur [F], $r = 0.869$, two-tailed, $p < 0.00001$. Pearson's Correlation, drinking water [F] vs. incisor [F], $r = 0.799$, two-tailed, $p < 0.00001$.

between inbred strains (Beck *et al.*, 2000).

The clinical presentation of dental fluorosis in mice parallels that which can be seen in mild forms of dental fluorosis in humans. Variations in the onset and severity of dental fluorosis among different inbred strains of mice when age, gender, food, housing, and fluoride exposure are rigorously controlled support the influence of genetic background on dental fluorosis susceptibility/resistance. Mice were grouped into resistant (129P3/J, FVB/NJ, CBA/J, and DBA/1J), intermediate (SWR/J, BALB/cJ, C57BL/10J, and DBA/2J), and sensitive (A/J, SJL/J/C3H/HeJ, and C57BL/6J) strains. Not all closely related strains behaved similarly. For example, the C57BL/6J and C57BL/10J strains diverged from a common ancestor ~ 70 yrs ago and show very similar profiles, whereas the DBA/1J and DBA/2J strains were separated more recently and show very different sensitivities. One might also view the differences between related strains as an indication of the actions of a small number of genes in determining susceptibility. The identification of dental-fluorosis-susceptible and -resistant inbred mouse strains will permit the genetic dissection of the genes and pathways

involved in enamel formation that underlie the pathogenesis of dental fluorosis. Presently, few studies have been conducted which explore an underlying genetic basis for fluoride resistance. High concentrations of fluoride have been used to isolate fluoride-resistant mutants of *Caenorhabditis elegans* which have led to the identification of a novel fluoride-resistant gene, an ion channel belonging to the degenerin/epithelial sodium channel superfamily, which regulates defecation rhythm (Katsura *et al.*, 1994; Take-Uchi *et al.*, 1998).

Quantitative light-induced fluorescence (QLF) has emerged in our studies as an innovative tool for the detection, staging, and quantification of dental fluorosis. Fluorescence of teeth and its implications in caries diagnosis have been studied (Angmar-Månsson *et al.*, 1996). Dental fluorosis has been observed on images during caries examinations with the QLF technique, presenting as diffuse dark areas (Ferreira-Zandona *et al.*, 2000). The increased fluorescence seen in the fluorosis lesion is most likely due to the increase in porosity that occurs in fluorosed enamel, similar to the increase in porosity that is found in caries (Angmar-Månsson *et al.*, 1994).

Our investigation of chronic fluoride burden in mineralized tissues (femur and mandibular incisor) in the study animals suggests that active removal of fluoride from the body is not a sufficient explanation for dental fluorosis resistance. Based upon the lack of significant differences between [F] in the erupted incisor and that in the inbred mouse strain ($p = 0.457$), it is possible that resistant mice are more tolerant to fluoride in their enamel organ microenvironment, whereas susceptible mice are more sensitive to the amount of fluoride within their enamel organ microenvironment. In the erupted mandibular incisors, enamel covers only the labial surface and averages less than 0.1 mm thick (Moinichen *et al.*, 1996). Therefore, it was not possible to separate the enamel from the dentin in our fluoride determinations and provide mapping of fluoride within the incisors' microanatomy.

Quantitative trait loci (QTL) mapping with the A/J (susceptible) and 129P3/J (resistant) inbred mouse strains can be used in this complex trait dissection to identify the actual genes that directly and/or indirectly contribute to an individual's susceptibility or resistance to develop dental fluorosis and to an understanding of the cellular roles and functions of these genes during the pathogenesis of fluorosis.

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