RESEARCH REPORTS
Biological

J.G. Carvalho[1], A.L. Leite[1], D. Yan[2], E.T. Everett[3], G.M. Whitford[4], and M.A.R. Buzalaf[1]*

[1]Department of Biological Sciences, Bauru Dental School, University of São Paulo, Al. Octávio Pinheiro Brisolla, 9-75, Bauru—SP 17012-901, São Paulo, Brazil; [2]Dental Research, University of North Carolina, Chapel Hill, NC, USA; [3]Department of Pediatric Dentistry and The Carolina Center for Genome Sciences, University of North Carolina, Chapel Hill, NC, USA; and [4]Department of Oral Biology and Maxillofacial Pathology, Medical College of Georgia, Augusta, GA, USA; *corresponding author, mbuzalaf@fob.usp.br

J Dent Res 88(11):1054-1058, 2009

Influence of Genetic Background on Fluoride Metabolism in Mice

ABSTRACT

A/J and 129P3/J mouse strains have different susceptibilities to dental fluorosis, due to their genetic backgrounds. This study tested whether these differences are due to variations in water intake and/or F metabolism. A/J (susceptible to dental fluorosis) and 129P3/J mice (resistant) received drinking water containing 0, 10, or 50 ppm F. Weekly F intake, excretion and retention, and terminal plasma and femur F levels were determined. Dental fluorosis was evaluated clinically and by quantitative fluorescence (QF). Data were tested by two-way ANOVA. Although F intakes by the strains were similar, excretion by A/J mice was significantly higher due to greater urinary F excretion, which resulted in lower plasma and femur F levels. Compared with 129P3/J mice given 50 ppm F, significantly higher QF scores were recorded for A/J mice. In conclusion, these strains differ with respect to several features of F metabolism, and amelogenesis in the 129P3/J strain seems to be unaffected by high F exposure.

KEY WORDS: dental fluorosis, fluoride metabolism, genetic susceptibility/resistance, inbred mouse strains, bone.

INTRODUCTION

Concurrent with the decline in dental caries, there has been an increase in the prevalence of dental fluorosis (Clark, 1994; Marthaler, 2004; Khan and Packer, 2006). While it is well-accepted that fluoride (F) interacts with mineralized tissues and, at elevated levels of intake, disturbs the mineralization process (Aoba and Fejerskov, 2002), the molecular mechanisms that underlie the pathogenesis of dental fluorosis remain unknown.

It has been suggested that genetic determinants influence an individual's susceptibility to develop dental fluorosis (Yoder *et al*., 1998). This hypothesis was tested in a mouse model system where genotype, age, gender, food, housing, and drinking water F-levels were under control (Everett *et al.*, 2002). Examination of 12 inbred strains of mice showed differences in susceptibilities to dental fluorosis. The A/J mouse strain was highly susceptible, with a rapid onset and severe development of dental fluorosis compared with the other strains tested, whereas the 129P3/J mouse strain was less affected, with minimal dental fluorosis. More recently, it has also been shown that these two strains have different bone responses to F exposure (Mousny *et al*., 2006).

The protocols for the studies mentioned above (Everett *et al.*, 2002; Mousny *et al*., 2006), however, did not include determination of F intake or excretion. Therefore, it was not known whether the "resistant" 129P3/J strain consumed less or excreted more F. We conducted this study to test the hypothesis that differences in susceptibilities to dental fluorosis are due to differences in water intake and/or F metabolism.

MATERIALS & METHODS

Mice

Weanling (3-week-old) male 129P3/J and A/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), and animals of the same strain were housed in pairs in plastic metabolic cages equipped with secondary cups that collected any spilled food and water, in the animal research facility at the Medical College of Georgia (MCG). The temperature and humidity in the climate-controlled room, which had a 12-hour light/dark cycle, were 23 ± 1ºC and 40-80%, respectively. The mice had free access to a low-F (0.95 mg/kg or ppm) diet (AIN76A, PMI Nutrition, Richmond, IN, USA). The research protocol was approved by the appropriate review boards of the MCG.

Fluoride Treatment

Initially, the mice were assigned to 3 groups based on the F concentrations in the drinking water (0, 10 or 50 ppm added as NaF). Each group consisted of 12 mice, 6 from each strain. During the first 2 wks, it was determined that the

DOI: 10.1177/0022034509347249

Received September 28, 2008; Last revision June 28, 2009; Accepted July 1, 2009

volume of water consumed by the A/J mice was significantly higher that by the 129P3/J mice. Thus, for the remainder of the study (7 wks) the F concentrations in the water given to the A/J mice were adjusted weekly in an attempt to equalize F intake by the 2 strains.

Body weights and food and water intakes were determined gravimetrically once each hr. In addition, 48-hr collections of urine and feces were made each hr so that the absorption and retention of F could be determined. The following equations were used: absorption = amount ingested – amount excreted in feces; retention = total ingested (food and water) – total excretion (urine and feces).

Sample Collection and Death

At the end of the study, when the mice were 12 wks old, they were anesthetized with ketamine and xylazine. A blood sample was collected from the heart into a lightly heparinized syringe for the determination of plasma F.

The right femurs were removed, cleaned of soft tissue, and frozen at -20ºC. Prior to F analysis, they were ashed at 600ºC overnight. The lower and upper jaws were removed and shipped to the University of North Carolina for the assessment of the degree of dental fluorosis according to clinical criteria and by quantitative fluorescence (QF).

Dental Fluorosis Phenotyping

Clinical Analysis

The determination of dental fluorosis was made clinically by two independent examiners for the entire lower incisor tooth surfaces, according to a modified TF index (Thylstrup and Fejerskov, 1978; Everett *et al.*, 2002).

Quantitative Fluorescence

The measure of fluorescence is a modification of the quantitative light-induced fluorescence (QLF) system that has been used to measure the severity of fluorosis in mice (Everett *et al.*, 2002). We used a modification of that technique, where the Inspektor hardware was replaced with a Nikon epifluorescence microscope equipped with a Chroma Gold 11006v2 set cube (exciter D360/40x, dichroic 400DCLP, and emitter E515LPv2). The lower incisors were removed from the mandible and allowed to remain slightly moistened. Teeth were viewed, labial side up and flat, on a black background at 2X magnification. Twelve-megapixel tiff images were acquired under standard exposure conditions. Images were analyzed with Image J software version 1.33u (http://rsb.info/nih.gov/ij/). Briefly, ten 300 x 300 pixel areas were randomly positioned over the pair of lower incisors and the mean grayscale values for each square determined. The average grayscale values for all 10 regions were then determined.

Fluoride Analysis

F concentrations in plasma, bone ash, and feces were determined after overnight hexamethyldisiloxane (HMDS)-facilitated diffusion (Taves, 1968; Whitford, 1996) with an ion-specific electrode (Orion Research, Beverly, MA, USA; Model 9409) and a calomel electrode (Accumet, Cambridge, MA, USA; Model 13-620-79), both coupled to a potentiometer (Orion Research, Model 720A).

F standards (0.0095 to 38 μg F) were prepared in triplicate and diffused in the same manner as the samples. In addition, non-diffused standards were prepared as the diffused standards, with exactly the same F concentrations. Comparison of the mV readings demonstrated that the F in the diffused standards had been completely trapped and analyzed (recovery > 95%). The mV potentials were converted to μg F based on a standard curve with a correlation coefficient of $r \geq 0.99$.

F concentrations in water and urine were determined with the electrode by the direct method after the samples were buffered with an equal volume of TISAB II. The standards ranged between 0.95 and 95.0 mg F/L.

Statistical Analysis

GraphPad Prism software version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for all the variables tested, except the clinical examinations. The assumptions of equality of variance (Bartlett's test) and normal distribution of errors (Kolmogorov-Smirnov test) were checked for all the variables tested (F intake, excretion, absorption, retention, and plasma and femur F concentrations). Since the assumptions were satisfied, two-way ANOVA and Bonferroni's *post hoc* test were carried out for statistical comparisons. The factors used were strain and treatment (water F concentration). Linear regression was also used to determine the relationship between plasma and femur F concentrations at the end of the metabolic study.

SPSS software version 11.0 for Windows (SPSS Inc. Headquarters, Chicago, IL, USA) was used for testing ordinal variables from the clinical data. The Spearman correlation test was used for the analysis of differences between the strains and among the treatments.

In all cases, the significance limit was set at 5%. The data are expressed as mean ± SE.

RESULTS

The mean (± SE) body weights of the A/J mice were 26.83 ± 0.32, 25.48 ± 0.49, and 27.24 ± 0.54 g for control, low-F, and high-F groups, respectively. 129P3/J mice presented similar values: 21.74 ± 0.29, 24.78 ± 0.46, and 23.86 ± 0.36 g, respectively.

A/J mice ingested nearly twice as much water as the 129P3/J mice during the 2 wks prior to starting the metabolic study and during the 7 wks of the metabolic study ($p < 0.001$). They also excreted more urine: 4.46 ± 0.18 *vs*. 3.89 ± 0.09 mL/48 hrs/cage ($p = 0.005$). The ratios of urine output to water intake during the 9 wks of the study were 0.34 ± 0.01 and 0.51 ± 0.01, respectively ($p < 0.0001$). Within the A/J strain, the mice in the low-F group ingested significantly more water than those in the control and high-F groups during the 7-week metabolic study, but less during the preceding 2 wks ($p < 0.05$) (Table 1).

The A/J mice ate more food, so their F intake from this source was significantly higher than that by the 129P3/J mice

Table 1. Water Intake Data during the 2 wks Prior to the Metabolic Study and during the 7-wk Metabolic Study

		Treatments*		
Water Intake	Strains	Control	Low	High
First 2 wks	A/J[A]	15.77± 0.52[a]	11.34 ± 0.69[b]	14.91 ± 0.53[a]
	129P3/J[B]	7.22 ± 0.39[a]	7.76 ± 0.65[a]	7.58 ± 0.55[a]
Last 7 wks	A/J[A]	12.17 ± 0.28[a]	14.50 ± 1.38[b]	12.16 ± 0.28[a]
	129P3/J[B]	7.97 ± 0.27[a]	7.86 ± 0.17[a]	7.57 ± 0.15[a]

* Mean ± SE. n = 6 for the first 2 wks and n = 21 for the last 7 wks. Unit: g/48 hrs/cage (2 mice/cage). Distinct uppercase superscripts indicate significant differences between the strains. Distinct lowercase superscripts in the same row indicate significant differences among the treatments.

Table 2. Fluoride Intake, Excretion, Absorption, and Retention during the 7 wks of the Metabolic Study

		Drinking Water Fluoride Levels		
	Strains	Control	Low [F]	High [F]
Intake				
Food	A/J[A]	14.76 ± 0.15[ab*]	14.38 ± 0.24[a]	15.28 ± 0.28[b]
	129P3/J[B]	11.45 ± 0.19[a]	11.72 ± 0.17[a]	11.73 ± 0.16[a]
Water	A/J	None[aA]	85.29 ± 4.41[bA]	345.48 ± 6.06[cA]
	129P3/J	None[aA]	78.62 ± 1.68[bA]	378.74 ± 7.70[cB]
Total	A/J	14.76 ± 0.15[aA]	99.67 ± 4.49[bA]	360.77 ± 6.15[cA]
	129P3/J	11.45 ± 0.19[aA]	90.34 ± 1.73[bA]	390.47 ± 7.73[cB]
Excretion				
Feces	A/J	6.39 ± 0.30[aA]	31.08 ± 1.37[bA]	129.64 ± 4.09[cA]
	129P3/J	4.70 ± 0.13[aA]	32.75 ± 1.01[bA]	145.57 ± 7.51[cB]
Urine	A/J	2.28 ± 0.14[aA]	23.03 ± 2.26[bA]	106.08 ± 5.46[cA]
	129P3/J	1.30 ± 0.10[aA]	6.91 ± 1.20[aB]	72.80 ± 5.59[bB]
Total	A/J	8.67 ± 0.37[aA]	54.11 ± 2.75[bA]	235.73 ± 6.60[cA]
	129P3/J	6.00 ± 0.16[aA]	39.67 ± 1.56[bB]	218.37 ± 8.81[cB]
Absorption	A/J[A]	8.36 ± 0.29[a]	68.58 ± 4.40[b]	231.12 ± 5.93[c]
	129P3/J[A]	6.75 ± 0.20[a]	57.59 ± 1.22[b]	244.90 ± 9.34[c]
Absorption (%)	A/J[A]	56.7 ± 1.93[a]	68.0 ± 1.59[b]	64.0 ± 1.10[b]
	129P3/J[A]	58.8 ± 1.21[a]	63.8 ± 0.79[a]	62.6 ± 1.87[a]
Retention	A/J	6.08 ± 0.33[aA]	45.56 ± 3.01[bA]	125.04 ± 6.43[cA]
	129P3/J	5.45 ± 0.21[aA]	50.67 ± 1.97[bA]	172.10 ± 8.49[cB]
Retention (%)	A/J[A]	41.3 ± 2.29[a]	45.4 ± 1.80[a]	34.6 ± 1.65[b]
	129P3/J[B]	47.4 ± 1.52[a]	56.0 ± 1.74[b]	44.0 ± 1.96[a]

* Mean ± SE (n = 21). Unit: μg/48 hrs/cage (2 mice/cage). Distinct uppercase superscripts indicate significant differences between the strains. Distinct lowercase superscripts indicate significant differences among the treatments.

($p < 0.001$). Within the A/J treatment groups, F intake with food was slightly but significantly higher in the high-F group than in the low-F group ($p < 0.01$) (Table 2).

F intake with water and total F intake were directly related to the water F concentrations ($p < 0.0001$). In the high-F treatment group, the 129P3/J mice ingested more F with water (and total intake) than the A/J mice ($p < 0.001$) (Table 2).

Fecal, urinary, and total F excretions were also directly related to the water F concentrations ($p < 0.0001$). Overall, total F excretion between the strains was not significantly different ($p = 0.071$). In the high-F group, fecal F excretion by the 129P3/J mice was higher than by the A/J mice. Urinary and total F excretions, however, were higher by the A/J mice in the low-F and high-F groups ($p = 0.003$) (Table 2).

The amounts of F that were absorbed, as well as the percentages of the ingested amounts that were absorbed by the 2 strains, did not differ significantly. Overall, significant differences in the amounts retained were found between the strains (129P3/J > A/J; $p < 0.0001$) and among the treatments ($p < 0.0001$). The percentages of ingested F that were retained by the 2 strains were also significant (129P3/J > A/J; $p < 0.001$) (Table 2).

Plasma and femur ash F concentrations were strongly related to the water F concentrations ($p < 0.0001$). The 129P3/J plasma concentrations were numerically higher than the A/J concentrations in each treatment group, but the difference was statistically significant only for the high-F group. The femur concentrations showed the same profile as that in plasma. Linear regression analysis relating plasma to femur F concentrations was highly significant ($r^2 = 0.632$, $p < 0.0001$) (Table 3).

There were significant differences in the clinical and QF data between the strains ($p = 0.007$ and $p = 0.0003$ for clinical and QF, respectively) and among the treatments ($p = 0.001$ and $p < 0.0001$ for clinical and QF, respectively). Despite the clinical evaluations being significantly different in both the low-F ($p = 0.001$) and high-F groups ($p = 0.003$), the QF data were significantly different only in the high-F group ($p < 0.001$) (Table 4). Despite the fact that the 129P3/J mice had higher F retentions and higher plasma and femur concentrations than the A/J mice (Tables 2 and 3), the expression of dental fluorosis in their incisors was less marked, although the difference was significant only in the high-F treatment group (Table 4).

DISCUSSION

This is the first in-depth fluoride metabolism study ever conducted with mice. A similar, 6-week study with weanling Sprague-Dawley rats was conducted in one of our laboratories (Whitford, 1991). The rats in Group E of that study received F in a manner similar to the mice in the present study, *i.e.,* they were fed a low-F diet (AIN76A) and fluoridated water. A comparison of the results from Group E with those of the present study indicates that these 2 species metabolized F quite differently. For example, according to the average values of the low-F and high-F groups of the present study, the mice and rats absorbed 63% and 89% of their ingested fluoride, they excreted 55% and 17% of their ingested F, and they retained 45% and 83% of their ingested F, respectively.

Table 3. F Concentrations in Plasma and Femur Ash

		Treatment		
		Control	Low [F]	High [F]
Plasma [F] (μg/L)	A/J	6.9 ± 0.4^{aA}	25.0 ± 3.7^{aA}	97.9 ± 7.8^{bA}
	129P3/J	9.0 ± 0.8^{aA}	31.0 ± 4.5^{aA}	143.4 ± 26.9^{bB}
Femur [F] (mg/Kg)	A/J	198.8 ± 11.7^{aA}	774.2 ± 100.0^{aA}	2890.9 ± 446.5^{bA}
	129P3/J	330.8 ± 43.4^{aA}	1075.3 ± 90.2^{bA}	3994.3 ± 314.2^{cB}

* Mean ± SE. n = 6. Distinct uppercase superscripts indicate significant differences between the strains. Distinct lowercase superscripts in the same row indicate significant differences among the treatments.

The metabolic characteristics of the 2 mouse strains in the present study also differed significantly in several respects. The main purpose of the present study was to determine whether such differences existed, and, if so, if they could at least partially explain the differences in susceptibility to dental fluorosis previously reported (Everett *et al.*, 2002). The first difference found was that the A/J mice ate more food and drank more water than the 129P3/J mice. Initially, we thought that this, by itself, could have accounted for the reported different susceptibilities to dental fluorosis (Everett *et al.,* 2002), since, in that study, the water F concentrations were unchanged, and food or water intake was not measured.

Because of the difference in water intake, we adjusted the F concentrations for the A/J mice on a weekly basis, in an attempt to provide similar amounts of F intake by the 2 strains. Our attempt was not entirely successful, but the only statistically significant difference was in the high-F group, where the 129P3/J mice ingested 7.4% more F than the A/J mice. Additionally, it should be noted that the higher water (and F) intake presented by the A/J mice in the first 2 wks might not have influenced the results of dental fluorosis, since mouse incisors have continuous growth and are completely renewed in 35-45 days (Zegarelli, 1944).

Other notable differences include the following:

(a) The A/J mice excreted a greater volume of urine than the 129P3/J mice, which was consistent with greater water intake, but the ratio of their urine output to water intake was 33% lower, which suggests a major difference in the metabolic handling of water.

(b) Despite similar or actually higher total F intakes, urinary and total F excretions by the 129P3/J mice were lower than those by the A/J mice. This difference was greatest in the low-F group for reasons that remain unknown. We determined that it was not due to problems with the cages, urine collection tubes, water bottles, or water bottle stoppers. The difference may have been due to a lower urinary pH, a major determinant of the renal clearance of F (Whitford, 1996), but this variable was not measured.

(c) The retention of F and the percentage of ingested F that was retained in the low-F and high-F groups were greater in the 129P3/J mice. This difference was reflected in their higher plasma and femur F concentrations, despite the fact that F intake by the A/J mice was much higher during the first 2 wks of the study, before we started adjusting their water F concentrations.

Table 4. Quantitative Fluorescence (QF) and Clinical Examination of Mouse Incisors (n = 5)

		Treatments		
		Control	Low [F]	High [F]
Strains	Animals	QF/Clinical	QF/Clinical	QF/Clinical
A/J	1	12.66/1	13.38/1	68.05/4
	2	10.35/1	12.83/1	55.47/4
	3	11.66/0	12.49/2	27.11/3
	4	11.60/0	14.67/2	31.54/3
	5	11.51/1	13.29/1.5	45.87/4
129P3/J	1	11.06/1	11.14/0	12.39/1
	2	12.06/1	11.60/0	17.57/1
	3	11.46/1	11.08/1	15.94/2
	4	12.11/1	11.22/0	15.95/2
	5	11.45/0	10.53/0	16.72/3
A/J	Total	11.56 ± 0.37^{aA}	13.33 ± 0.37^{aA}	45.61 ± 7.55^{bA}
129P3/J	Total	11.63 ± 0.20^{aA}	11.1 ± 0.17^{aA}	15.71 ± 0.88^{aB}

Distinct uppercase superscripts indicate significant differences between the strains. Distinct lowercase superscripts in the same row indicate significant differences among the treatments for the QF data.

With regard to the latter point, it would have been reasonable to expect higher femur F concentrations in the A/J mice than in the 129P3/J mice in the study by Everett *et al*. (2002), because both strains were given water with the same F concentrations, assuming that their A/J mice consumed more water, as noted in the present study. The femur concentrations, however, were lower, although not significantly so. These higher F concentrations were observed, however, in the whole incisors (enamel and dentin) of the A/J mice given 50 ppm F in the water. Thus, there

is the possibility that the pattern of F uptake in the different types of hard tissues (tooth and bone) is not equal in these strains.

Despite these differences in the metabolic handling and tissue concentrations of F, the clinical and QF findings confirm that the teeth of the 129P3/J mice are more resistant to the effects of F (Everett *et al.*, 2002). The physiological, biochemical, and/or molecular mechanisms underlying this resistance remain to be determined.

ACKNOWLEDGMENTS

The authors thank Ms. Aarti Arun for her fluoride analyses and Prof. Fábio Sampaio for aid with the statistical analysis. This study was supported by FAPESP (2005-04671-0), CNPq (471485/2007-1), and NIH/NIDCR (DE-014853). This study was based on a thesis to be submitted to Bauru Dental School, University of São Paulo (Brazil), in partial fulfillment of the requirements for the PhD degree in Oral Biology.

REFERENCES

Aoba T, Fejerskov O (2002). Dental fluorosis: chemistry and biology. *Crit Rev Oral Biol Med* 13*:*155-170.

Clark DC (1994). Trends in prevalence of dental fluorosis in North America. *Community Dent Oral Epidemiol* 22*:*148-152.

Everett ET, McHenry MA, Reynolds N, Eggertsson H, Sullivan J, Kantmann C, *et al*. (2002). Dental fluorosis: variability among different inbred mouse strains. *J Dent Res* 81*:*794-798.

Khan A, Packer NH (2006). Simple urinary sample preparation for proteomic analysis. *J Proteome Res* 5*:*2824-2838.

Marthaler TM (2004). Changes in dental caries 1953-2003. *Caries Res* 38*:* 173-181.

Mousny M, Banse X, Wise L, Everett ET, Hancock R, Vieth R, *et al.* (2006). The genetic influence on bone susceptibility to fluoride. *Bone* 39*:* 1283-1289.

Taves DR (1968). Determination of submicromolar concentrations of fluoride in biological samples. *Talanta* 15*:*1015-1023.

Thylstrup A, Fejerskov O (1978). Clinical appearance of dental fluorosis in permanent teeth in relation to histologic changes. *Community Dent Oral Epidemiol 6:*315-328.

Whitford GM (1991). Fluoride, calcium and phosphorus metabolism in the rat: comparison of 'natural ingredient' with semipurified diets. *Arch Oral Biol* 36*:*291-297.

Whitford GM (1996). Metabolism and toxicity of fluoride. *Monogr Oral Sci* 16(Rev 2):1-153.

Yoder KM, Mabelya L, Robison VA, Dunipace AJ, Brizendine EJ, Stookey GK (1998). Severe dental fluorosis in a Tanzanian population consuming water with negligible fluoride concentration. *Community Dent Oral Epidemiol* 26*:*382-393.

Zegarelli EV (1944). Adamantoblastomas in the Slye stock of mice. *Am J Pathol* 20:23-87.