RESEARCH REPORTS
Biological

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J Dent Res 90(6):788-792, 2011

Assessment of Dental Fluorosis in *Mmp20*$^{+/-}$ Mice

ABSTRACT

The molecular mechanisms that underlie dental fluorosis are poorly understood. The retention of enamel proteins hallmarking fluorotic enamel may result from impaired hydrolysis and/or removal of enamel proteins. Previous studies have suggested that partial inhibition of *Mmp20* expression is involved in the etiology of dental fluorosis. Here we ask if mice expressing only one functional *Mmp20* allele are more susceptible to fluorosis. We demonstrate that *Mmp20*$^{+/-}$ mice express approximately half the amount of MMP20 as do wild-type mice. The *Mmp20* heterozygous mice have normal-appearing enamel, with Vickers microhardness values similar to those of wild-type control enamel. Therefore, reduced MMP20 expression is not solely responsible for dental fluorosis. With 50-ppm-fluoride (F^-) treatment *ad libitum*, the *Mmp20*$^{+/-}$ mice had F^- tissue levels similar to those of *Mmp20*$^{+/+}$ mice. No significant difference in enamel hardness was observed between the F^--treated heterozygous and wild-type mice. Interestingly, we did find a small but significant difference in quantitative fluorescence between these two groups, which may be attributable to slightly higher protein content in the *Mmp20*$^{+/-}$ mouse enamel. We conclude that MMP20 plays a nominal role in dental enamel fluorosis.

KEY WORDS: protease/proteinase, gene expression, protein expression, fluoride, enamel.

DOI: 10.1177/0022034511398868

Received August 18, 2010; Last revision December 21, 2010; Accepted December 21, 2010

INTRODUCTION

Matrix metalloproteinase-20 (MMP20; enamelysin) is a predominantly tooth-specific MMP (Turk *et al*., 2006) that is essential for proper dental enamel formation. Homozygous mutation of human *MMP20* causes the non-syndromic enamel malformation termed amelogenesis imperfecta (Kim *et al*., 2005; Ozdemir *et al*., 2005; Papagerakis *et al*., 2008; Lee *et al*., 2010). Mice harboring targeted disruption of both *Mmp20* alleles have severely impaired enamel matrix protein processing, leading to hypoplastic enamel with a decreased mineral content, decreased enamel hardness, and malformed enamel that fractures from the dentin surface (Caterina *et al*., 2002; Bartlett *et al*., 2004).

Proteolytic enzyme activity in dental enamel was demonstrated almost four decades ago (Suga, 1970). Rat tooth sections at the secretory stage of development were placed in a gelatin containing photographic emulsion, and proteolytic activity was observed by the presence of cleared areas where the gelatin had been digested. Hydrolysis of gelatin by the enamel layer was completely inhibited by the presence of 5 mM NaF (Suga, 1970). Thus, concurrent with the first observation of proteolytic activity within secretory-stage enamel, the first investigation of the effect of fluoride (F^-) on this activity was also assessed. Subsequently, several studies have investigated fluoride's action on enamel matrix proteolytic activity. Those studies have shown that F^- inhibits maturation-stage protease activity (DenBesten and Heffernan, 1989), inhibits MMP20 activity (DenBesten *et al*., 2002), and inhibits *Mmp20* transcription (Zhang *et al*., 2006, 2007). Conversely, one study demonstrated that neither proteolytic activity from the secretory stage nor that from the maturation stage was inhibited by up to 10 mM NaF exposure (Gerlach *et al*., 2000), and we performed enzyme kinetic analysis to demonstrate that concentrations of up to 10 mM F^- did not inhibit recombinant MMP20 activity (Tye *et al*., 2010). We also previously demonstrated, by use of qPCR on rat secretory-stage incisor enamel organs, that *Mmp20* expression was not affected after 6 wks of exposure to 0, 50, 100, or 150 ppm F^- as NaF in drinking water (Sharma *et al*., 2010).

Here, we applied a genetic approach to assess whether reduced *Mmp20* expression levels contribute to the etiology of dental fluorosis. We compared the level of fluorosis observed in F^--treated wild-type mice with that observed in F^--treated *Mmp20*$^{+/-}$ mice. We reasoned that if *Mmp20* expression plays a substantial role in dental fluorosis, *Mmp20*$^{+/-}$ mice would be more susceptible to the adverse effects of F^- than would wild-type mice. Prior experimental approaches were correlative in nature by asking if reduced MMP20 expression or activity correlated with severity of dental fluorosis. However, in this

in vivo approach, we directly assess if reduced *Mmp20* expression leads to increased fluorosis.

MATERIALS & METHODS

Mice

All handling, care, and usage of animals were approved by The Forsyth Institute. Mice were housed in an AAALAC-approved facility. Six-week-old C57BL/6 wild-type and backcrossed *Mmp20*$^{+/-}$ mice were divided into 4 groups (n = 5) and received either 0 ppm or 50 ppm F^- in their drinking water for 6 wks. Fluoride content of drinking water was measured with a fluoride ion-selective electrode. Mouse chow contained an average of 1.57 ppm F^-.

Determination of *Mmp20* Expression Levels

Quantitative real-time RT-PCR (qPCR) of first mandibular molar enamel organ RNA extracted from 7-day-old mice (6 wild-type, 9 *Mmp20*$^{+/-}$, and 5 *Mmp20*$^{-/-}$ mice) was used to determine relative expression levels of *Mmp20* as a function of the stably expressed internal reference control gene *Eef1α1,* as previously described (Pfaffl, 2001; Kubota *et al*., 2005). Primers were: *Mmp20* forward (5′-GAGACACACATTTCGACAATGCTGAG-3′), *Mmp20* reverse (5′-GTTGGGTACATCAGTGCTGATGGAT-3′); and *Eef1α1* forward (5′-ATTCCGGCAAGTCCACCACAA-3′, *Eef1α1* reverse 5′-CATCTCAGCAGCCTCCTTCTCAAA-3′).

For MMP20 protein content analysis, hard tissues from mandibular first molars from 6 mice (n = 3/genotype) were incubated in 1 mL of 0.5% formic acid at 4°C overnight, centrifuged briefly to remove residual insoluble material, transferred to an Ultracel 3K filter (3-kDa cut-off, Millipore, Billerica, MA, USA), and centrifuged. The enamel protein on the filter was eluted into sample buffer. Approximately 40 μg of protein was run on SDS-PAGE and transferred to a membrane, and MMP20 bands were visualized by Western blotting with an antiserum to MMP20 (Abcam, Cambridge, MA, USA; Ab39037, 1:2500 in TBST).

Determination of Water, Plasma, Bone, and Urine F^- Concentrations

Plasma and bone ash F^- concentrations were determined after overnight hexamethyldisiloxane (HMDS)-facilitated diffusion (Taves, 1968; Whitford and Reynolds, 1979). Urine was collected from anesthetized mice *via* bladder puncture prior to their death, and fluoride concentration was determined with a F^- ion-selective electrode. Incisors were collected for assessment of dental fluorosis and microhardness values.

Measurement of Dental Fluorosis (TF) and Quantitative Fluorescence (QF)

Two blinded examiners performed independent assessments of dental fluorosis status for each animal (inter-examiner Kappa, 0.850; $p < 0.001$). The determination of dental fluorosis was made clinically over the entire lower incisor tooth surfaces according to a modified Thylstrup-Fejerskov (TF) index (Thylstrup and Fejerskov, 1978; Fejerskov *et al*., 1994; Everett *et al*., 2002).

A quantitative fluorescence (QF) system was previously devised to evaluate the severity of fluorosis in mice (Everett *et al*., 2002). A Nikon epifluorescence microscope equipped with a Chroma Gold 11006v2 set cube (exciter D360/40x, dichroic 400DCLP, and emitter E515LPv2) was used to capture fluorescent images of teeth. The fluorescence was converted to grayscale values, and intensities were analyzed with Image J software version 1.33u (http://rsb.info/nih.gov/ij/).

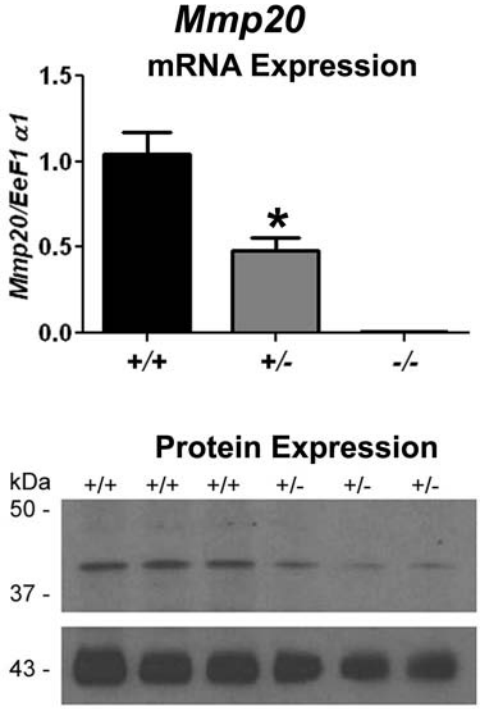

Figure 1. Expression levels of MMP20 in *Mmp20*$^{+/+}$, *Mmp20*$^{+/-}$, and *Mmp20*$^{-/-}$ mice. For analysis of *Mmp20* transcript expression, first mandibular molars were extracted from 6 wild-type, 9 heterozygous, and 5 null mice. **(Top)** Quantitative real-time PCR (qPCR) analysis demonstrated that expression levels in the heterozygous mice were approximately half those observed in the wild-type mice (*$p < 0.001$). The null mice did not express *Mmp20*. Analyses were performed in triplicate, and error bars represent standard error of the mean. **(Bottom)** Western blot analysis demonstrated a significant reduction in MMP20 protein level within enamel extracted from heterozygous mice as compared with the protein levels from wild-type control mice. Upper band, MMP20; lower band, β-actin.

Vickers Microhardness Testing

Erupted portions of mandibular incisors from wild-type and *Mmp20*$^{+/-}$ littermate mice were washed and dehydrated with graded alcohol and acetone. Incisors were embedded sagittally in hard-formulation epoxy embedding medium (EpoFix, EMS, Hatfield, PA, USA). Samples were ground and polished to 0.25 μm with diamond suspensions (EMS). The polished samples were tested for enamel microhardness on an M 400 HI testing machine (Leco, St. Joseph, MI, USA). Testing was performed with a load of 25 g for 5 sec with a Vickers tip. Twenty indentations *per* sample were performed on 3 teeth *per* group and averaged.

Statistical Analysis

We evaluated statistical significance by performing a two-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test,

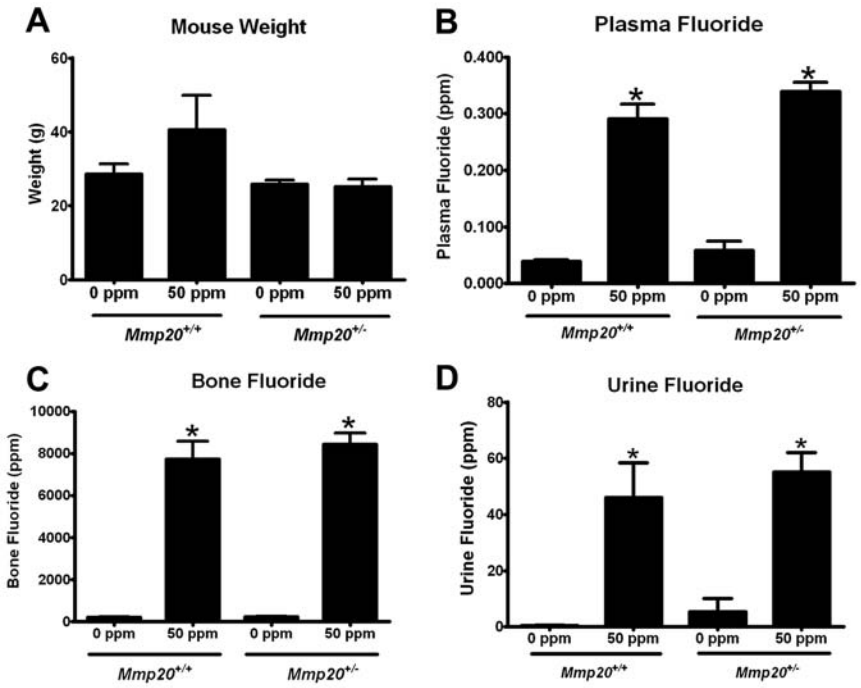

Figure 2. Mouse weight and tissue fluoride levels in untreated (0 ppm) or fluoride-treated (50 ppm) *Mmp20* wild-type and heterozygous mice. **(A)** The mean (± SE) body weights in grams were 28.61 ± 2.71 (wild-type, 0 ppm), 40.58 ± 9.37 (wild-type, 50 ppm), 25.77 ± 1.14 (heterozygous, 0 ppm), and 25.11 ± 2.11 (heterozygous, 50 ppm). A two-way analysis of variance revealed no significant difference in weight among the groups. **(B)** The means (± SE) of plasma fluoride levels in ppm were 0.0388 ± 0.0035 (wild-type, 0 ppm), 0.2912 ± 0.0258 (wild-type, 50 ppm), 0.0582 ± 0.0165 (heterozygous, 0 ppm), and 0.3388 ± 0.0166 (heterozygous, 50 ppm). **(C)** The means (± SE) of bone fluoride levels in ppm were 217.5 ± 8.9 (wild-type, 0 ppm), 7718.0 ± 863.6 (wild-type, 50 ppm), 239.3 ± 9.8 (heterozygous, 0 ppm), and 8432.0 ± 541.7 (heterozygous, 50 ppm). **(D)** The means (± SE) of urine fluoride levels in ppm were 0.495 ± 0.025 (wild-type, 0 ppm), 46.020 ± 12.420 (wild-type, 50 ppm), 0.675 ± 0.195 (heterozygous, 0 ppm), and 55.100 ± 7.030 (heterozygous, 50 ppm). Except for panel D, each bar in each panel of Fig. 2 represents an average of the same 5 mice *per* treatment group. The number of mice represented in each bar in panel D are 2, 4, 3, and 2 for the $Mmp20^{+/+}$ 0 ppm and 50 ppm, and $Mmp20^{+/-}$ 0 ppm and 50 ppm treatment groups, respectively. Overall, *Mmp20* heterozygosity had no effect on fluoride tissue levels in untreated or fluoride-treated mice. *$p < 0.0001$.

using GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). Inter-examiner Kappa was calculated by use of SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Quantification of MMP20 Expression in the Secretory-stage Enamel Organ

To determine if *Mmp20* heterozygous mice have reduced *Mmp20* expression, we performed qPCR on mRNA from first molars of seven-day-old wild-type, heterozygous, and homozygous null mice. Both primers annealed to an area (catalytic domain) deleted from wild-type mice to generate the *Mmp20* null mouse. This precluded detection of mRNA encoding a non-functional protein. The results demonstrated that the heterozygous mice expressed half the quantity of *Mmp20* transcripts, and the homozygous null mice expressed no *Mmp20* transcripts, when compared with the transcript levels observed in the wild-type mice (Fig. 1, top). Western blotting confirmed that MMP20 protein levels were also reduced by approximately half in the heterozygous mouse enamel (Fig. 1, bottom). Thus, no compensatory mechanism is present to increase expression of *Mmp20* in the heterozygous mice.

Analysis of Tissue F^- Concentrations among Mouse Treatment Groups

After 6 wks of F^- treatment, the mice were weighed, and bone (femur), urine, and plasma were collected for analysis of F^- levels. The mice did not significantly differ ($p = 0.146$) in weight among genotypes and F^- treatment groups (Fig. 2A). Plasma and bone F^- levels (Figs. 2B, 2C) were significantly increased in the F^--treated mice compared with those in the untreated mice ($p < 0.0001$). However, within each fluoride treatment group, there was no significant difference in tissue fluoride levels between genotypes. This was the same for the urine analyses (Fig. 2D), except that the significance level was lower ($p < 0.05$), because urine could not be obtained from all available mice. In all cases, tissue F^- levels between genotypes within the same F^- treatment group were not significantly different (Fig. 2). Therefore, each treatment group of mice received a similar overall F^- dose during the six-week treatment time.

Assessment of Dental Fluorosis (TF) and Quantitative Fluorescence (QF)

By use of a modified TF scale (Fig. 3A), we demonstrated that a significant increase in dental fluorosis occurred for both mouse genotypes when mice were treated with 50 ppm F^- *vs.* the 0 ppm controls ($p < 0.0001$). However, for each F^- treatment group, dental fluorosis between genotypes was not significantly different (TF scale). The QF method measures enamel fluorescence that correlates highly to the severity of dental fluorosis. Incisor enamel QF in the wild-type or heterozygous mice treated with 50 ppm F^- was significantly higher when compared with that of the 0 ppm controls ($p < 0.0001$). The 0 ppm F^- controls were not significantly different between genotypes. However, there was a small but statistically significant difference ($p < 0.05$) in QF between the F^--treated $Mmp20^{+/+}$ and $Mmp20^{+/-}$ mice (Fig. 3B). Also shown are pictures of teeth representative of those observed in our experiments from each mouse genotype and from each F^- treatment group (Fig. 3C).

Analysis of Enamel Hardness as a Function of Genotype and F^- Treatment

We performed Vickers microhardness testing on enamel from wild-type and *Mmp20* heterozygous mice with or without F^-

exposure to determine if *Mmp20* heterozygotes have softer-than-normal dental enamel. For both genotypes, microhardness of F^--treated enamel significantly decreased ($p < 0.0001$) as compared with that of controls (Fig. 4). No significant differences were observed between genotypes within their respective F^- treatment group. These results indicate that while F^- exposure results in softer enamel, the lack of an *Mmp20* allele does not by itself significantly affect enamel hardness.

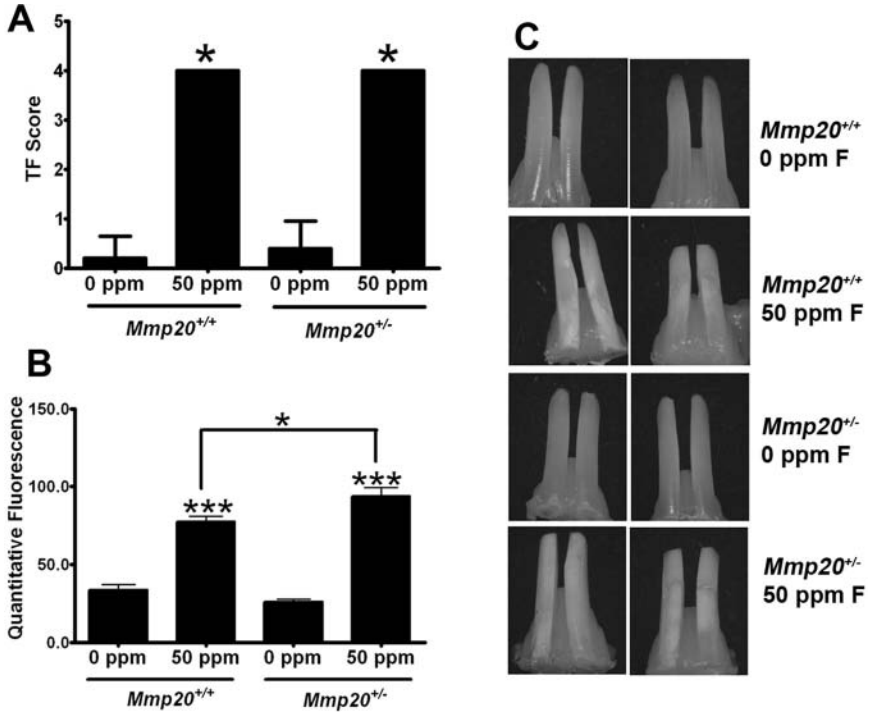

Figure 3. Assessment of dental fluorosis in *Mmp20* wild-type and heterozygous mice. **(A)** All mice of both genotypes treated with 50 ppm fluoride received a TF score of 4. One mouse in the wild-type 0 ppm group received a score of 1, and 2 mice from the heterozygous 0 ppm group received a score of 1. All others received a 0 score. A two-way ANOVA followed by Bonferroni's *post hoc* test revealed highly significant differences, but no difference existed between genotypes within a fluoride treatment group. **(B)** The means (± SE) of quantitative fluorescence (QF) values from mandibular incisors were 27.34 ± 3.3 (wild-type, 0 ppm), 65.12 ± 2.94 (wild-type, 50 ppm), 19.42 ± 1.66 (heterozygous, 0 ppm), and 78.24.0 ± 3.90 (heterozygous, 50 ppm). The observed differences between the 0 ppm and 50 ppm F^- treatment groups were highly significant ($p < 0.0001$). No significant difference existed between the wild-type and heterozygous 0 ppm F^--treated mice. However, there was a small but statistically significant difference between the wild-type and heterozygous 50 ppm F^--treated mice. The heterozygous mice had slightly greater fluorescence values ($p < 0.05$). **(C)** Representative mouse mandibular incisors from *Mmp20* wild-type and heterozygous mice displaying normal (0 ppm F^-) and fluorotic dental enamel (50 ppm F^-). *$p < 0.05$; ***$p < 0.0001$.

DISCUSSION

Previously we demonstrated in rodents (rats) that 6 wks of exposure to 0, 50, 100, or 150 ppm F^- as NaF in drinking water does not affect *Mmp20* mRNA levels (Sharma *et al*., 2010). This study demonstrated that mice with one mutated *Mmp20* allele express approximately half the normal levels of MMP20, that tissue levels of F^- are unaffected by *Mmp20* heterozygosity, that *Mmp20*$^{+/-}$ mice have normal-appearing enamel as determined by TF score and quantitative fluorescence, that the F^--treated *Mmp20*$^{+/-}$ mouse enamel has more fluorescence than the wild-type mouse, that F^- treatment results in softer-than-normal enamel, and that no difference in enamel hardness exists between the mouse genotypes within a F^- treatment group.

A dose of 10 μM NaF was previously demonstrated to reduce MMP20 protein expression by 21.0% in cultured cells and was also demonstrated to inhibit an *Mmp20* promoter-reporter construct by 35% (Zhang *et al*., 2007). While this may occur *in vitro*, our results generated *in vivo* show that loss of an *Mmp20* allele with an associated 50% reduction in MMP20 expression does not cause a fluorosis-like phenotype. Tooth color was normal, and no difference in enamel hardness was observed between the genotypes. Therefore, dental fluorosis cannot be solely attributed to partial reduction of *Mmp20* expression.

The only significant phenotype associated with decreased MMP20 expression was an increased average QF value in F^--treated *Mmp20*$^{+/-}$ incisors. To determine if the increased fluorescence was caused by a rougher enamel surface in *Mmp20*$^{+/-}$ mice compared with wild-type mice, we performed atomic force microscopy in contact mode with a silicon nitride tip. However, we found no significant difference in roughness between genotypes (data not shown). Although we have not directly compared the sensitivity of QF *vs*. microhardness values, it is possible that QF is a more sensitive fluorosis indicator than are microhardness determinations. This may account for the discrepancy in results between these two techniques. We did look at the EDTA soluble protein-rich zone of unerupted incisor enamel in wild-type mice and observed high QF values. These QF values decreased once the enamel erupted in its virtually protein-free form (unpublished results). Thus, enamel fluorescence may positively correlate with protein content. It is possible, therefore, that the F^--treated *Mmp20*$^{+/-}$ mice retain slightly more enamel protein than do the wild-type controls. Perhaps a few enamel matrix proteins remain uncleaved by MMP20 in *Mmp20*$^{+/-}$ enamel, and these larger proteins cannot be easily re-absorbed in conditions that cause dental fluorosis. Further studies are necessary to confirm this theory.

Recently, we demonstrated that F^- induces the phosphorylation of the α-subunit of eukaryotic translation initiation factor, eIF2, in both mice and rats during the maturation stage of enamel formation (Sharma *et al*., 2008, 2010). eIF2α phosphorylation is associated with transient attenuation of global protein translation.

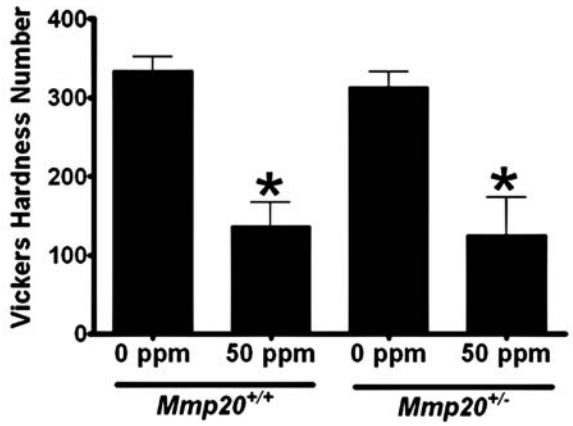

Figure 4. Quantification of enamel hardness in *Mmp20* wild-type and heterozygous mice. The bar chart represents Vickers microhardness data for enamel from *Mmp20*$^{+/+}$ and *Mmp20*$^{+/-}$ mice with (50 ppm) or without (0 ppm) F^- exposure. The Y-axis shows Vickers hardness number (VHN). The means (± SE) of microhardness levels in Vickers hardness numbers (VHN) were 334.0 ± 17.85 (wild-type, 0 ppm), 136.6 ± 30.02 (wild-type, 50 ppm), 312.8 ± 19.78 (heterozygous, 0 ppm), and 125.6 ± 48.03 (heterozygous, 50 ppm). For each genotype, the 50 ppm F^--treated mice had significantly lower microhardness values than did the 0 ppm mice. However, within each F^- treatment group, there was no significant difference in hardness levels between genotypes. *$p < 0.0001$.

An overall decrease in protein translation would be expected to reduce the quantity of protein necessary for routine cell activities. Therefore, F^--mediated eIF2α phosphorylation and subsequent reduction of protein translation are consistent with the F^--induced delay (30%) in the modulation cycle of maturation-stage rat ameloblasts between their ruffle-ended and smooth-ended morphologies (Smith *et al*., 1993). In addition, F^- also suppresses the transcription of maturation-stage genes in the rat, including *Klk4* and *Amtn* (Sharma *et al*., 2010). Our current study supports the existing hypotheses stating that a delay in the removal of matrix proteins during the maturation stage is responsible for the increased protein content of fluorosed enamel (DenBesten, 1986). In any case, this study demonstrates that enamel fluorosis cannot be solely attributed to a reduction of *Mmp20* expression.

ACKNOWLEDGMENTS

We thank Dr. Henry Margolis and Amy Litman for their enamel surface roughness determinations. This work was supported by NIDCR grants DE018106 (JDB), DE16276 (JDB), and DE018104 (ETE).

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