INSULIN SIGNALLING AND OXIDATIVE STRESS IN THE MECHANISM UNDERLYING STREPTOZOTOCIN AGGRAVATED DENTAL FLUOROSIS

Jingchun Sun,a Yan Wang,b Xiuhua Yu,c Hui Xu,b,*
Changchun, People’s Republic of China

ABSTRACT: The aim of this study was to elucidate whether insulin status affected the severity of dental fluorosis by characterizing changes in incisor morphology and oxidative stress status in vivo and analyzing the insulin receptor expression in osteoblasts exposed to the fluoride ion (F) in vitro. The development of drinking water-type dental fluorosis, with or without streptozotocin (STZ), was studied in 60 Wistar rats. The F-treated rats received F in low or high doses, 10 and 20 mg F/kg body weight per day by gavage for 2 months, respectively, and STZ in a single dose of 52 mg/kg bw by intraperitoneal injection. The results indicated that the F-treatment induced dental fluorosis which was significant aggravated by the deficiency of insulin activity mediated by the STZ administration. The low dose F-treatment, with 10 mg F/kg body weight per day significantly reduced the activity of glutathione peroxidase (GPX) and the combination of 20 mg F/kg body weight per day and STZ inhibited the activity of superoxide dismutase (SOD). No significant change occurred in the malondialdehyde (MDA) levels with the F or STZ treatments. In the in vitro experiment, a low dose of 2 mg F/L stimulated the protein expression of the insulin receptor, while at a high dose of 16 mg F/L inhibition occurred. The results indicate that insulin and its intracellular signaling are involved in the development of dental fluorosis and that the process may also involve the occurrence of oxidative stress.

Keywords: Dental fluorosis; Glutathione peroxidase; Insulin; Insulin signalling; Malondialdehyde; Oxidative stress; Streptozotocin; Superoxide dismutase.

INTRODUCTION

Although the fluoride ion (F) has been applied topically and, in some countries, added to drinking water, to reduce dental caries,1 its presence in drinking water can result in various types of morbidity including dental and skeletal fluorosis.2 Thus, environmental F is considered to be a double-edged sword. More research is required to gain a greater understanding of the mechanisms by which dental fluorosis is produced. The early-maturation stage of enamel formation appears to be particularly sensitive to F.3 Experiments involving animals exposed to excessive F have also been observed to have changes in insulin secretion, thus indicating a relationship between insulin and fluorosis.4 Our previous experiment demonstrated that excessive F interfered with insulin secretion and sensitivity, and that a significant decrease of insulin sensitivity occurred in rats treated with the combination of F and streptozotocin (STZ).5 A close relationship has been demonstrated between oral disease and increased blood glucose in diabetes mellitus.6 Yeh et al. found that Akita -/- mice at 6 weeks of age showed chalky white incisors which correlated with hyperglycemia.7 To the present date, there
has been little literature on the role of insulin in the mechanism of dental fluorosis. The aims of the present study were to study the relationship of insulin and dental fluorosis and whether oxidative stress was involved by (i) determining whether STZ aggravated dental fluorosis, (ii) investigating the role of oxidative stress status in the mechanisms of fluorosis and diabetes, and (iii) observing the intracellular insulin receptor expression in osteoblasts exposed to varying concentrations of F.

**METHODS AND MATERIALS**

**Animals and treatment:** Sixty Wistar rats (male, 150–180 g) were purchased from the Experimental Animal Center of Bethune Medical College of Jilin University. A standard pellet diet and tap water were supplied ad libitum. All the experimental protocols related to using and treating the animals were approved by the Ethics Committee on the Use and Care of Animals of Jilin University (Changchun, People’s Republic of China). The 60 rats were divided into six groups (n = 10 per group): (1) normal control group, (2) 10 mg F/kg bw per day group (low dose of F group), (3) 20 mg F/kg bw per day group (high dose of F group), (4) STZ group (STZ control group), (5) STZ+10 mg F/kg bw per day group (STZ+low dose of F group), (6) STZ+20 mg F/kg bw per day group (STZ+high dose of F group). The administration of the sodium fluoride (NaF) (Sigma-Aldrich, USA) was by gavage daily. The streptozotocin (STZ) was administered as a single dose of 52 mg STZ/kg bw given by intraperitoneal (ip) injection one month after the commencement of the experiment. Forty-eight hours after the STZ injection, the blood glucose was measured by tail-vein sampling using a Blood Glucose Meter (Roche Co.), to check that those that received STZ had blood glucose readings of ≥11.1 mmol/L. The control rats were administered normal saline by gavage and ip injection. The experiment, including the treatment with F in groups 2, 3, 5, and 6, was for a period of 2 months in total. At the end of the two months, all the rats were euthanized. Blood was collected and the serum separated for the analysis of the oxidative stress status.

**Lipid peroxidation assay:** The serum malondialdehyde (MDA) level was tested with 100 µL of serum. The MDA values were calculated from the molar extinction coefficient of the MDA–thiobarbituric acid (TBA) complex. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX) were each determined with 200 µL of serum. Three parameters were measured with kits purchased from the Nanjing Jiancheng Institute of Bioengineering (Chinese) according to the manufacturer’s instructions.

**Cell culture and treatment:** The MC3T3-E1 cell line (Cell Bank of the Chinese Academy of Sciences) has been isolated from C57BL/6 mouse calvaria and is used widely as a cell model for osteoblast differentiation. In these experiments, MC3T3-E1 cells were cultured in α-modified Eagle’s medium (α-MEM) (Hyclone), supplemented with 10% fetal bovine serum and a penicillin/streptomycin mixture (100 U penicillin G/mL+100 g streptomycin sulfate/mL) at 37°C with CO₂. The NaF was administered to investigate the action of F on osteoblastic cells. The experiment included three F-treated groups and one control
α-MEM group. At the end of the experiment, the cells were fixed for the respective analyses.

**Immunofluorescent analysis of insulin receptor expression:** The MC3T3-E1 cells were plated in 24-well trays at a density of $1 \times 10^5$ cells per well and treated with α-MEM containing 2, 8, or 16 mg F/L for 2 days. Immunofluorescent analysis for MC3T3-E1 cells was performed according to a modified procedure. Briefly, the cell slides were blocked with 10% H$_2$O$_2$ at room temperature for 10 min, followed by treatment with 3% bovine serum albumin (BSA) at room temperature for 30 min. The slides were incubated in the dark with goat polyclonal insulin receptor (Santa Cruz, 1:100) diluted in 0.1 M phosphate buffered saline (PBS) overnight at 4°C, and further incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibodies (Santa Cruz, 1:1000) at room temperature for 1 hr. They were then stained with 4, 6-diamidino-2-phenylindole (DAPI). The slides were fully rinsed with 0.1 M PBS between the individual steps.

**Statistics analysis:** All the values of the samples were illustrated as the mean±standard deviation. The significant differences were analyzed using one-way analysis of variance (ANOVA) with SPSS 18.0 software (SPSS Inc, Chicago, USA) for Windows, and p<0.05 level was considered a significant difference.

**RESULTS**

**Gross incisor morphology of the rats treated with fluoride:** This study indicated that the incisor teeth were sensitive to exposure to excessive F for one month. Compared to control teeth (Figure 1a), treatment with 10 mg F/kg bw per day for one month caused a partial loss in the lower incisors of the normal transparency or translucency of the glossy creamy white-light brown enamel (Figure 1b). The loss of transparency was more obvious in the rats treated with 20 mg F/kg bw for a period of 1 month with a greater proportion the lower incisors appearing white (Figure 1c).

![Figures 1a, b, and c. Gross incisor morphology of rats treated with excessive fluoride for 1 month. The Wistar rats were treated with fluoride by gavage. a: incisor morphology of control group, b: incisor morphology of 10 mg F/kg bw per day group, c: incisor morphology of 20 mg F/kg bw per day group. n=10 for each group.](image)

Treatment with 10 mg F/kg bw per day for 2 months still caused only a partial loss of transparency of the incisors (Figure 2b), while treatment with 20 mg F/kg bw per day for two months caused more characteristics of dental fluorosis to appear with a chalky white opaque appearance and brown striations (Figure 2c).
The combination treatment of F and STZ, for 2 months, produced more severe alterations in the appearance of the incisors compared to treatment with F or STZ alone (Figures 2 and 3).

Combined treatment with STZ+10 mg F/kg bw/day caused an obvious chalky white change in the whole incisor (Figure 3b), and treatment with STZ+20 mg F/kg bw/day induced brittle fractures of the incisors accompanied by a chalky white appearance with brown striations (Figure 3c). These data indicate that the surface changes of the incisors depend on the dose and duration of F exposure, and that the administration of STZ aggravated the dental changes.

Serum oxidative stress status of the rats treated with fluoride: In the present study, the activity of glutathione peroxidase (GPX) in the 10 mg F/kg bw per day group was significantly lower than that in the control group (Figure 4a). The level of malondialdehyde (MDA) showed no significant changes in the F-treated groups (Figure 4b). The activity of SOD was significantly decreased in the 20 mg F/kg bw per day+STZ group compared to the STZ control group (Figure 4c). The decreases in SOD and GPX indicated the occurrence of increased lipid peroxidation.
Figures 4a, b, and c. Effects of combined treatment with fluoride and streptozotocin on the oxidative stress status in the serum of the rats. The Wistar rats were treated with F by gavage and streptozotocin by intraperitoneal injection and divided into 6 groups: control, 10 mg F/kg bw/day, 20 mg F/kg bw/day, STZ; STZ+10 mg F/kg bw/day, and STZ+20 mg F/kg bw/day. The quantitative analysis of (a) glutathione peroxidase (GPX), (b) malondiadehyde (MDA) levels, and (c) superoxide dismutase (SOD) levels were performed with biochemical tests. The results are expressed as mean±SD. n = 8 for each group. Compared to the respective control groups: *p<0.05, †p<0.01.
Protein level of the insulin receptor in the MC3T3-E1 cells: The effect of F on the protein expression of the insulin receptor in vitro was observed. In Figure 5, the insulin receptor antibody, labeled by fluorescein-isothiocyanate, is shown in green and the DNA, labeled by Hoechst33342, in blue. Differences occurred in the intensity of the fluorescence staining of the insulin receptors in the MC3T3-E1 cells after exposure to F for 2 days. Compared to the control group (Figure 5a), the fluorescence intensity of the positive staining cells was obviously higher in the 2 mg F/L group (Figure 5b). However, there were fewer positively staining cells in the 16 mg F/L group, and the fluorescence intensity also decreased (Figure 5d). These results demonstrated a low dose of F (2 mg F/L) had a stimulatory action on the insulin receptor protein in the MC3T3-E1 cells while a high dose of F (16 mg F/L) had an inhibitory action.

Figures 5a, b, c, and d. Representative images for the protein expression of the insulin receptor in MC3T3-E1 cells exposed to fluoride with immunofluorescence staining. The insulin receptor antibody labelled by fluorescein-isothiocyanate is shown in green and the DNA labelled by Hoechst33342 in blue (red arrows). Under microscopy (original magnification × 400), the cells showed fluorescence intensity changes for the insulin receptor staining in their cytoplasm (red arrows) from the control group. a: control group, b: 2 mg F/L group, c: 8 mg F/L group, and d: 16 mg F/L group.

DISCUSSION

Teeth are the target organ in dental fluorosis and the F- and SZT-treated rats in the present study showed the characteristic appearances of dental fluorosis in the affected tooth enamel with a loss of transparency, the development of opaque chalky white areas, and the occurrence of brown striations. A F level in drinking
water of 0.7–1.2 ppm (mg/L) has been recommended for the reduction of tooth decay with toxicity occurring with F intakes of 5–10 mg F/kg bw. However, a new recommendation, dated 24 April 2015, by the US Department of Health and Human Services Federal Panel on Community Water Fluoridation now recommends an optimal F concentration in drinking water of 0.7 mg F/L and the Country Standards for India and Senegal, West Africa, are 1 mg F/L and 0.6 mg F/L, respectively, with a rider to the India limit being the “lesser the fluoride the better, as fluoride is injurious to health.” The systemic ingestion of high levels of F in early childhood can lead to the enamel defects of dental fluorosis and some authorities now consider that F is more effective in the treatment of dental caries when applied topically to the teeth rather than being ingested in drinking water.

In 1999, DenBesten found that dental fluorosis results in a subsurface hypomineralization of the enamel and contributes to a delay in the removal of amelogenins at the early-maturation stage of enamel formation. The timing of these stages varies with the type of tooth. Therefore, the changes in the outward appearance of a tooth are likely to have been caused by the alteration of the tooth structures such as the enamel and dentine. The incisors are the teeth most sensitive to developing dental fluorosis, the appearance of which can include white specks, white or colored striations, and rough and pitted surfaces. In the current study, discoloration with loss of transparency was observed in the incisors of rats exposed to an excessive amount of F for 1 month with the high dose of F causing a larger discolored area in the teeth than occurred with the low dose of F. The changes were consistent with clinical dental fluorosis, in which the degree of discoloration depends on the dose of F, the duration of the F exposure, and the timing of the exposure. With increased F exposure time, the severity of dental fluorosis increased from mild to moderate. After 2 months of treatment, partial discoloration of the teeth with a loss of transparency appeared in the incisors of rats treated with the low dose of F (10 mg F/kg bw per day) and more marked changes, with extensive loss of transparency, a chalky white appearance, and brown striations, occurred in the teeth of the high dose of F-treated rats (20 mg F/kg bw per day). In comparison, the severity of the dental fluorosis was more prominent in the rats treated with combination of F+STZ. White mottling and brown striations were present in the incisors of the rats treated with the low dose of F+STZ, and these changes were accompanied by increased tooth brittleness and fractures in the high dose of F+STZ-treated rats. It is known that the incisors of rats continue to develop throughout the whole of the rat’s life, and hence the teeth of rats remain susceptible to developing dental fluorosis with exposure to excessive F throughout the life span. The severity of dental fluorosis increased in the rats treated with the combination of F+STZ compared to those treated with F alone.

Furthermore, the in vitro experiment showed that the protein expression of the insulin receptor was enhanced in osteoblastic cells exposed to low doses of F (2 mg F/L), suggesting that F evoked intracellular insulin signaling in osteoblasts. Our previous results demonstrated that excessive F affected insulin secretion and sensitivity, and that insulin sensitivity was reduced in rats treated with the
combination of F+STZ. Linked with the above results, the STZ administration probably interfered with the insulin signaling of the osteoblast in the cementum by inhibiting insulin activity and thereby aggressively influenced the development of dental fluorosis.

Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system in an organ or organism. It is recognized that oxidative stress is involved in the mechanism underlying F induced injuries in vitro in several types of cells and also in vivo in tissues in people and animals. Numerous investigations have shown that the underlying mechanism involves elevated levels of free radicals and attenuated antioxidant defenses. Moreover, F can alter the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) in MC3T3-E1 cells and lead to an imbalance of the redox state or oxidative stress. In the present study, after treatment for 2 months, a significant decrease of GPX enzyme activity occurred with the low dose F-treatment but the changes in the serum MDA level and the SOD activity were not significant. The combined treatment with F+STZ reduced the activity of GPX but not to a significant extent and the administration of STZ as a single dose had no appreciable effect on GPX activity. In contrast, the activity of SOD was increased with the administration of STZ as a single dose and was reduced with the high dose F+STZ treatment compared to the STZ control. Damage to pancreatic β-cells after the administration of STZ by generating free radicals has been reported. It has also been reported from in vivo studies that rats treated with STZ showed oxidative stress as reflected by increased serum MDA concentrations. In the present study, STZ administration hardly impaired the redox balance to a significant extent but the combined treatment with F+STZ significantly decreased the activity of SOD and the low dose F-treatment significantly decreased the GPX activity. These data suggest that the redox balance and the activity of the antioxidant enzymes were affected in the F-treated animals and that the administration of STZ further exacerbated this.

CONCLUSION

The data from the present study indicate that prolonged exposure to a high concentration of F is deleterious to the gross morphology of the incisor teeth of rats with the development of dental fluorosis and that this is aggravated by impaired insulin receptor signaling. F exposure impaired the redox balance in vivo, and the inhibition of the activity of antioxidant enzymes was significant in the rats treated the combination of F+STZ. The data indicate that insulin signaling is involved in the mechanism of dental fluorosis and that oxidative stress may also play a part.

ACKNOWLEDGEMENTS

This work was supported by a grant for skeletal fluorosis research from the National Natural Science Foundation of China (81673111), the Norman Bethune Program of Jilin University (20122222), and the Doctoral Fund of the Ministry of Education of China (20130061110084).
CONFLICTS OF INTEREST STATEMENT
The authors declare that they have no conflicts of interest.

REFERENCES
11 Evans RW, Stamm JW. An epidemiologic estimate of the critical period during which human maxillary central incisors are most susceptible to fluorosis. J Public Health Dent 1991;51:251-9