SUMMARY: The aims of this study were to examine the effect of fluoride (F) on the expressions of transforming growth factor TGF-$\beta$1 and the signal transduction protein Smad2/3 in ameloblasts of rat incisors, and to explore the antagonistic effect of melatonin. Forty Wistar rats were randomly divided into 6 groups: sodium fluoride (NaF) solution drinking groups (3 groups) and NaF solution drinking combined with intraperitoneal administration of melatonin groups (3 groups). After 10 weeks, hematoxylin and eosin (HE) staining and immunohistochemistry staining were used to examine the morphology of ameloblasts of rat incisors and TGF-$\beta$1 and Smad2/3 expressions. A micrographic system and image analysis software were used to measure the mean optical density (OD) of the positively stained samples from each group. The results showed that the rats in the NaF solution drinking groups developed typical symptoms of dental fluorosis, and both TGF-$\beta$1 and the intracellular signal transduction factor Smad2/3 were positively expressed in ameloblasts. The expression levels in the experimental groups were significantly lower than those of the control groups. There was no significant difference in the expressions of the different factors between the melatonin administered groups and the groups without melatonin administration. Overall, excessive F inhibits TGF-$\beta$1 and Smad2/3 secretion, and disturbs normal signal transduction between the epithelium and mesenchyme. No antagonistic effect of melatonin on the formation of dental fluorosis was found in this experiment.

Keywords: Ameloblast; Dental fluorosis in rats; Melatonin; Smad2/3; TGF-$\beta$1.

INTRODUCTION

Fluoride (F) has not been demonstrated to be a necessary trace element in the human body or required for the development of healthy teeth or bones. The long-term intake of F in excessive amounts can lead to chronic fluorosis, mainly manifested by damage to the teeth and bones. Dental fluorosis is the earliest clinical manifestation of chronic fluorosis. Whereas the cause of this disease is clear, its exact mechanism still remains elusive.

Melatonin (MT) is a type of indole hormone widely distributed in living organisms. As a synchronizer for the biological clock of the human endocrine system, MT shows a wide range of biological activities and participates in many physiological activities, including those in the central nervous system, reproductive system, endocrine system and immune system. Studies have shown that MT is highly lipophilic and is therefore highly dispersible and able to permeate cell membranes. It is an antioxidant and is able to inhibit oxidation in the cell membrane, cytoplasm and nucleus. MT can effectively remove oxyradicals from chemical reactions, and increase the activity of antioxidant enzymes. It can therefore effectively protect biological macromolecules, such as lipids and DNA,
from being damaged by free radicals, and protect the organism from the damage caused by oxyradicals.\(^4\)

A large amount of clinical data and animal experiments have shown that chronic fluorosis can cause cells in animal tissues such as liver, brain, kidney, and bone to produce free radicals.\(^3\)–\(^6\) MT can protect brain tissue from the oxidative stress response caused by fluorosis. However, until now, there have been no reports on whether MT can intervene in dental fluorosis caused by chronic fluorosis.

In this study, a rat model for dental fluorosis was established to examine the effect of \(F\) on the expressions of TGF-\(\beta\)1 and the signal transduction protein Smad2/3 in ameloblasts of rat incisors, and to further explore the mechanism of dental fluorosis. By comparing the expressions of TGF-\(\beta\)1 and Smad2/3 in \(F\) intake groups, with and without MT administration, this study also examined whether MT played an antagonistic role in the formation of dental fluorosis, and thus provided new insight into the clinical prevention of dental fluorosis.

**MATERIALS AND METHODS**

**Grouping of experimental animals:** Forty 4-week old Wistar rats (20 males and 20 females; weighing 60–70 g) were used in the experiment. All the rats were numbered by weight in descending order, and a random numbers table was used to randomly divide them into 6 groups as follows: negative control group A (8 rats, drinking distilled water), low \(F\) group B (7 rats, drinking NaF solution made with distilled water, 60 mg \(F^{-}/L\)), high \(F\) group C (7 rats, drinking NaF solution made with distilled water, 120 mg \(F^{-}/L\)), low \(F + \) MT group D (6 rats, drinking NaF solution made with distilled water, 60 mg \(F^{-}/L\) + intraperitoneal administration of MT), high \(F + \) MT group E (6 rats, drinking NaF solution made with distilled water, 120 mg \(F^{-}/L\) + intraperitoneal administration of MT), and the MT control group F (6 rats, drinking NaF solution made with distilled water + intraperitoneal administration of saline). The NaF solutions, at all concentrations, were freely provided for drinking. MT (5 mg/mL) was administered intraperitoneally at a 5 mL/kg body weight dose between 16:00 h and 17:00 h every day from the fifth week. As a MT control, 1 mL saline was injected. The temperature of the feeding room was kept at 22–25°C, and food was provided for free intake.

**Sample processing and hematoxylin and eosin (HE) staining of the histological slices:** At week 10, the animal was killed after being anesthetized by ether. Complete bilateral mandibles were removed and the tissues were processed so that mandibular incisors and part of the mandible surrounding the mandibular incisors were left. The sample was rinsed with 0.01% PBS and then placed in 4% paraformaldehyde for fixation at 4°C for 30 h. Next, the sample was decalcified in 10% EDTA-2Na solution at 4°C; the decalcification solution was changed every 3 days. After 6 weeks, the sample was sliced and subjected to serial ethanol dehydration, vitrification by dimethylbenzene, and paraffin imbedding. Continuous mesiodistal slices along the longitudinal axis of the tooth, and each slice was 6 µm thick. Then the slices were mounted to poly-L-lysine treated slides, heated at 65°C for 3 hr and marked for future use.
HE staining: Routine HE staining was performed, and the morphology of ameloblasts during the development of rat incisors in each group was examined under a light microscope.

**Immunohistochemistry staining and results evaluation:** Routine immunohistochemistry training was performed, and the slices were subjected to paraffin slicing, dewaxing and hydration at room temperature. Then they were incubated with 3% H$_2$O$_2$ for 10 min at room temperature to deactivate the endogenous peroxydase. The slices were placed in a box with citric acid buffer and heated for 2.5 min in a microwave until boiling. After the slices were cooled to room temperature, a few drops of normal calf serum blocking buffer was added and they were incubated at room temperature for 20 min. 1:100 (PBS) TGF-$\beta$1 or Smad2/3 primary antibody (Santa Cruz Inc., USA) was added for incubation at 4°C overnight. Then, after incubation at room temperature for 30 min, the slices were rinsed with PBS for 2 min 3 times. A few drops of biotinylated secondary antibody were added for incubation at 37°C for 25 min. The slices were rinsed with PBS for 2 min 3 times. A few drops of SABC were added for incubation at 37°C for 25 min. The slices were rinsed with PBS for 5 min 4 times. A chromogenic reaction was induced using a DAB chromogenic reagent, the time of which was adjusted under the microscope in order to adjust the degree of color change. The reaction was stopped by a water rinse and the slices were subjected to counterstaining by hematoxylin for 2 min. They were then rinsed with water, subjected to color separation by 1% HCl-ethanol for several seconds and bluing by clear water for 10 min, serial alcoholic dehydration, vitrification by dimethylbenzene, and a neutral balsam seal. For negative control, the primary antibody solution was replaced by PBS. If brown granules showed in the cytoplasm or cell nuclei, the staining was considered as positive.

A MetaMorph imaging system was used to collect immunohistochemistry staining results to transform the intensity of positive expression to digital signals and perform semi-quantitative analysis on the staining intensity of the positive areas. For each slice at high magnification, positive expression analysis on randomly selected 5 non-overlapping views of the same developmental stage was performed. The mean optical density (OD) of positively stained cells was measured. A smaller OD indicated smaller tissue content and vice versa. Relative evaluation was performed based on a comparison of staining intensity.

To maximally avoid interference from human factors and assure comparability among slices, when making the slices, the conditions at steps, such as immunohistochemistry staining, were kept the same as much as possible. All measurements were completed at one time, during which the illumination intensity and microscope magnification remained fixed.

**Statistical analysis:** SPSS 12.0 statistics software was used for data processing. An analysis of variance (ANOVA) was performed on the OD values of immunohistochemistry results from all groups. The results are presented as mean±SD. Differences were considered significant at p<0.05.
RESULTS

Observation of incisor morphology of rats in each group: The incisors of rats in the control group developed well. The tooth surface was of a brown yellow color and was smooth and semi-transparent. For the low FF group, the incisor surface was non-transparent in some of the areas and exhibited slight roughness with regular, fine horizontal stripes of interleaved brown and white colors. For the high F group, the incisor surface was notably rough, exhibiting changes to a chalk color, and the horizontal stripes of interleaved brown and white colors were more striking. These changes were typical symptoms of dental fluorosis although no notable substantive defect was observed. There were no significant differences between the weights of rats in the control groups and those of rats in the experimental groups (Figure 1).

HE staining results:

CONTROL GROUP: Columnar ameloblasts in the secretory phase were evenly arranged as a single layer. The cell nuclei were distant from the basement membrane and the enamel matrix was secreted, while Tomes processes entered deep into the enamel matrix on the near ameloblast side. In the mature phase, Tomes processes disappeared, and the height and width of ameloblasts gradually decreased as the secretion of the enamel matrix decreased until the enamel matured.

LOW F GROUP: The ameloblasts were arranged irregularly and the height of the columnar shape was decreased compared to the controls. There was vesiculation between the cells.

HIGH F GROUP: The decrease in the height of the columnar shape was even more substantial, and the arrangement was disorganized. Vesiculation was common. The enamel matrix secretion was decreased and the enamel was thin (Figure 2).
Immunohistochemistry staining results:

TGF-β1 expression in ameloblasts: TGF-β1 was expressed in the cytoplasm and nuclei, and the expression was strong during the secretory and mature phases of ameloblasts. It was positively expressed in the newly formed enamel matrix, the odontoblastic layer, the dental pulp, the stellate reticulum and the stratum intermedium. The TGF-β1 expressions in the low F and high F groups were both significantly lower than that in the control group (p<0.05). There was no
significant difference in TGF-β1 expression between the low F and high F groups (p>0.05; see Figures 3 and 4, and Table 1).

![Figure 3](image1.png)

**Figure 3.** The expression of TGF-β1 in ameloblasts (SABC, 40×10). A: control group; B: low F group; C: high F group; D: dentin group. od: odontoblasts; pd: predentin; d: dentin; E: emanel; Am: ameloblasts; Sl: stratum intermedium; Sr: stellate reticulum.

![Figure 4](image2.png)

**Figure 4.** The expression of TGF-β1 in the ameloblasts of rats from different groups.
SMAD2/3 EXPRESSION IN THE AMELOBLASTS: For the control group, Smad2/3 expression was strongly positive during the secretory and mature phases of the ameloblasts. It was positively expressed in the odontoblastic layer, the stellate reticulum and the stratum intermedium. The Smad2/3 expressions in the low F and high F groups were both significantly lower than that in the control group (p<0.05). There was no significant difference in Smad2/3 expression between the low F and high F groups (p>0.05; see Figures 5 and 6, and Table 1).

Table 1. OD values of positively expressed TGF-β1 and Smad2/3 in the ameloblasts
Values are mean±SD

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>TGF-β1</th>
<th>Smad2/3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group A</td>
<td>8</td>
<td>0.1613±0.0019</td>
<td>0.2277±0.0054</td>
</tr>
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<td>Low fluoride group B</td>
<td>7</td>
<td>0.1582±0.0026**</td>
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<td>High fluoride group C</td>
<td>7</td>
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<td>0.2146±0.0042**</td>
</tr>
</tbody>
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Compared with the control group, *p<0.05, **p<0.01,

Figure 5. Smad2/3 expression in the ameloblasts of rats from different groups (SABC, 40×10).
b: blank control group; A: control group; B: low F group; C: high F group.
Am: ameloblast; SI: stratum intermedium; Sr: Stellate reticulum.
THE ANTAGONISTIC EFFECT OF MELATONIN ON DIFFERENT FACTORS: The expressions of MMP-20, TIMP-2, TGF-β1 and Smad2/3 were basically the same between groups with and without melatonin administration. There were no significant differences (Figures 7, 8, and Table 2).
Fluoride-induced TGF-β1 and Smad2/3 expression in the ameloblasts of rat incisors and the effect of melatonin
Zhang, Zhang, Cheng

**DISCUSSION**

As signal molecules, the TGF-β superfamily plays an important role in a series of processes including tooth germ development, differentiation of ameloblasts and odontoblasts, and the formation of tooth hard tissues. Bègue-Kirn et al. reported that TGF-β1 can promote the synthesis of the extracellular matrix of tooth germ ameloblasts, and induce cell differentiation. Coin et al. cultured isolated enamel organs *in vitro* and found that pre-ameloblasts did not polarize and acquire properties of functional ameloblasts until TGF-β1 was added. Zhu et al. used *in situ* hybridization to analyze the expression and localization of TGF-β1 mRNA during the development of a mouse tooth. They found that it was expressed locally in the tooth epithelium at the bud and cap stages and largely expressed in the ameloblast layer and dental papilla cells at the bell stage. TGF-β1 expression increased with the differentiation of odontoblasts and ameloblasts. It has been proposed that TGF-β1 plays an important role during tooth formation. Fan et al. also reported that cells in the stratum intermedium could synthesize TGF-β1, possibly to regulate the initiation of ameloblast differentiation. Experiments using a mouse dental fluorosis model showed that, at the bell stage, TGF-β1 expression was mainly localized in the stratum intermedium, stellate reticulum and ameloblasts. There was also some TGF-β1 expression in the dental papilla cells and odontoblasts. It was speculated that TGF-β1 participated in the terminal differentiation of ameloblasts.

In the current study, it was found that TGF-β1 was positively expressed in cells in the stellate reticulum and stratum intermedium, suggesting that TGF-β1 takes part in the formation of early tooth morphology and cell differentiation during the early stage of tooth germ development. TGF-β1 expression is positive in ameloblasts and odontoblasts at the secretory and mature stages, suggesting that TGF-β1 may be closely related to the differentiation of ameloblasts and

**Table 2.** OD values of positively expressed TGF-β1 and Smad2/3 in the ameloblasts of rats from groups with and without MT administration. Values are mean±SD

<table>
<thead>
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<td>0.2146±0.0042**</td>
</tr>
<tr>
<td>Low fluoride + MT group D</td>
<td>6</td>
<td>0.1585±0.0012**</td>
<td>0.2182±0.0052**</td>
</tr>
<tr>
<td>High fluoride + MT group E</td>
<td>6</td>
<td>0.1569±0.0008**</td>
<td>0.2152±0.0812**</td>
</tr>
<tr>
<td>Control + saline group F</td>
<td>6</td>
<td>0.1609±0.0009 *</td>
<td>0.2269±0.0117 *</td>
</tr>
</tbody>
</table>

Compared with the control group, *p<0.05, **p<0.01,
odontoblasts and matrix formation. This is consistent with the results reported by Su et al.\textsuperscript{12} A study by Cheng et al. also showed that at the early bell stage, TGF-β1 expression was strongly positive in the inner enamel epithelium, enamel knot, stratum intermedium and dental lamina, whereas it was positive in the outer enamel epithelium.\textsuperscript{13} At the bell stage, TGF-β1 expression was strongly positive in pre-ameloblasts, ameloblasts, pre-odontoblasts and odontoblasts. Tsuchiya et al. reported that the TGF-β1 expression in the enamel organ at the mature stage was significantly higher than at the early secretory stage.\textsuperscript{14} Elevated TGF-β1 expression also led to an increase in the expression of the corresponding stress response factors in the early enamel organ and a decrease in the expression of apoptosis-inhibiting cytokine Bcl-2/Bax. It was thus proposed that TGF-β1 may play an important role in the apoptosis of ameloblasts at the mature stage of enamel development.

Previous studies have shown that F can inhibit the differentiation of ameloblasts, and the synthesis and secretion of the matrix, yet the underlying mechanisms remain unclear. TGF-β1 plays an important role in inducing the differentiation of ameloblasts and odontoblasts and synthesis of the extracellular matrix. The question is, does excessive F cause dental fluorosis through TGF-β1? A study by Wang et al. found that low-dose (20 mg/L) and medium-dose (50 mg/L) F significantly inhibited TGF-β1 expression in the enamel organ of the mouse incisor.\textsuperscript{15} The current study examined the effect of F on TGF-β1 expression in the ameloblasts of rat incisors using an immunohistochemistry method to preliminarily investigate the mechanism of dental fluorosis. Our results showed that TGF-β1 expression in the experimental groups was significantly lower than that in the control group. This reduced TGF-β1 expression indicated that F inhibited TGF-β1 expression, suggesting that F may disturb ameloblast differentiation and matrix secretion and thus may cause an impediment to enamel development via inhibiting TGF-β1 expression.

The Smads family is the intracellular signal transduction molecules for TGF-β. In 1996, Eppert et al. screened Smad2 from a human kidney cDNA library and demonstrated that Smad2 was a specific signal transduction molecule downstream from the TGF-β1 receptors.\textsuperscript{16} He also showed that Smad2 could specifically transduce the TGF-β1 signal into the nuclei to a functional state.\textsuperscript{17} Bao et al. found that Smad3 was expressed during all stages of tooth germ development.\textsuperscript{18} Masahiko et al. reported that the enamel of Smad3-knockout mice showed changed to a chalk color and showed insufficient mineralization compared to wild type mice\textsuperscript{19} suggesting that Smad3 may play an important role in enamel formation and may influence enamel mineralization.

In the current study, changes in Smad2/3 expression in the ameloblasts under the influence of low-dose and high-dose F were examined, and the effect of F on tooth germ development and ameloblast differentiation was investigated to explore the cause of dental fluorosis. The results showed that, in the control group, Smad2/3 expressions were strongly positive in the ameloblasts at the secretory and mature stages. Smad2/3 was also positively expressed in the stellate reticulum, the stratum
intermedium and the odontoblastic layer, and weakly expressed in the dental pulp cells. Thus, we reasoned that Smad2/3 may be involved in the secretion of the enamel matrix. It influences the secretion of the enamel matrix components, such as amelogenin, and is involved in enamel maturation and mineralization. Under the influence of F, Smad2/3 expression was reduced, suggesting that excessive F may inhibit Smad2/3 expression during the development of rat incisors. F may disturb the translocation of TGF-β superfamily signals from the cytoplasm to nuclei via inhibiting the activity of Smad2/3. Or, as F decreased the molecular expression of Smad2/3, the cellular function was influenced and ameloblast differentiation and normal enamel mineralization were inhibited. This may be the intracellular mechanism of dental fluorosis. However, further studies are needed to verify this hypothesis and clarify the details.

F is not one of the trace elements necessary for the human body and excessive intake makes it an exogenous toxin that stimulates cells to produce oxy-radicals. Many studies have shown that, in chronic fluorosis, soft tissue injury is closely related to free radicals, and it has been proposed that the damage caused by free radicals is a key component of the mechanism of chronic fluorosis. It has also been shown that cartilage damage is closely related to free radicals. Yu et al. used electron-spin-resonance (ESR) to detect the free radical content in the bones of rats exposed to fluorosis, and found that the free radical content in the bone tissues was significantly increased, accompanied by thinning of the cortical bone, narrowing of the bone trabeculae, and a decrease in the number of bone cells. It has been proposed that excessive F accumulation could cause imbalance in the antioxidation system and an increase in free radicals in the bones, which then leads to bone damage. Free radicals mainly impair the membrane structures in cells and cell organelles. F can cause an increase in the free radical content in tissues and thus impair the cellular structure, causing tissue damage.

In 1993, Tan et al. for the first time, reported the antioxidative activity of MT, which appeared to be a highly effective endogenous free radical scavenger, with an ability to scavenge free radicals 4 times that of glutathione, 14 times that of mannitol, and 2 times that of vitamin E.

Rao et al. demonstrated that MT can clear free radicals caused by excessive fluoride. However, whether MT plays a protective role against damage caused by dental fluorosis has not been reported. In the current study, preliminary exploration was conducted by examining the potential differences in TGF-β1 and Smad2/3 expressions caused by intraperitoneal administration of MT when the rats exhibited mild symptoms of dental fluorosis. The aim was to investigate whether MT showed an antagonistic effect after dental fluorosis had occurred. The results showed that there were no significant differences between the groups with and without MT administration. It may be possible that MT cannot repair damage that has already been caused by dental fluorosis, or MT does not play an antagonistic role by influencing the expression of factors, including TGF-β1 and Smad2/3. This issue merits further study to elucidate the detailed mechanisms.
ACKNOWLEDGMENT

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REFERENCES

21 Xu P. Free radicals and fluoride poisoning induced cartilage injury. Foreign Medical Sciences (Section of Medgeography) 2000;21(3):97-100.
26 Rao MV, Thakur SB. Effects of melatonin and amla antioxidants on fluoride-induced genotoxicity in human peripheral blood lymphocyte cells. Fluoride 2013;46(3):128-34.