DOWN-REGULATION OF EXPRESSION OF TYPE 1 COLLAGEN GENE IN DENTIN OF FLUORIDE EXPOSED RATS

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ABSTRACT
Excessive ingestion of fluoride leads to abnormal mineralization of both enamel and dentin. Mechanisms about enamel fluorosis have been clarified. The knowledge about dentin fluorosis is limited and the mechanism is unclear. The present experiment was aimed to ascertain the excessive ingestion of fluoride on collagen metabolism in dentin of incisor teeth of rats. Rats (n=12) were divided into two groups. Rats of group I served as control and received only tap water and those of group II received sodium fluoride @ 200 mg/L of drinking water ad lib for a period of 90 days. Quantitative real-time polymerase chain reaction was used to quantify the gene expression levels of Col1a1 in dentin. Significant decreases in expression of Col1a1 gene in the dentin of incisor teeth of fluoride exposed rats was noted at the end of the experiment.

Key words: Colla1 gene, Dentin, Fluoride, Rats.

INTRODUCTION
Dentin is the most abundant dental tissue and largely determines the size and shape of teeth. Dentin is formed by odontoblast cells. The unique structure and composition of dentin allow it to function as the substructure for the rigid enamel tissue, thereby imparting teeth with the ability to flex and absorb tremendous functional loads without fracturing. On a weight basis, mature dentin is about 70% mineral, 20% organic matrix and 10% water (Linde and Goldberg, 1993). Type I collagen, the major organic component expressed in dentin (Linde et al., 1980), consists of two $\alpha_1$(I) and one $\alpha_2$(I) polypeptide chains that assemble into the functional collagen protein. The $\alpha_1$(I) and $\alpha_2$(I) subunits are encoded as Col1a1 and Col1a2, respectively, and have different primary amino acid sequences (Miu et al., 2002; Xu et al., 2003).

Susheela (1970) suggested that collagen protein was a target damaged by excessive fluoride (F) ingestion (Susheela and Sharma, 1982). Since then, disturbance of collagen metabolism in F exposed animals has been noted in studies showing that fluoride decreased the biosynthesis of collagen protein and increased the catabolism of collagen (Susheela and Mukerjee, 1981; Bely et al., 1988; Wardas et al., 2002). Many studies have shown that fluoride can negatively affect collagen metabolism and leads to the breakdown of collagen in bone, tendon, muscle, skin, cartilage, lung, kidney and trachea (Susheela and Jha, 1981; Susheela and Mukerjee, 1981; Pu et al., 1996). Despite the wealth of knowledge on the effect of F on collagen metabolism, there is relatively little information on the collagen gene expression in normal and fluorosed dentin. Therefore, elucidation of its molecular mechanism is crucial to understanding this aspect of F toxicity.

Real-time RT-PCR (reverse transcription polymerase chain reaction) has enabled the quantification of mRNA with high accuracy, reproducibility, and sensitivity over a wide dynamic range without the need for post-PCR processing (Gibson et al., 1996). Here, with the help of real-time RT-PCR, we quantified the expression levels of the Col1a1 gene, which encodes the type I collagen of dentin of experimental rats.
MATERIALS AND METHODS
Animals and experimental design: The experiment was performed on growing female wistar albino rats (n=12) weighing about 100 gm with approval from Institute (IVRI) Animal Ethics Committee. Animals were housed in polypropylene cages in 12 h dark/12 h light cycles with temperature of the laboratory animal house ranging from 18 to 25°C and humidity between 55 and 60%. Rice bran was used as the bedding, which was changed during cleaning of cages on every alternate day. The animals were provided with laboratory animal feed, procured from the Feed Unit of the Institute, approximately at the dose of 15 g/rat daily. The left over feed on the next day was discarded and fresh feed was provided to them. Water was supplied ad lib.

After 15 days of acclimatization period, they were randomly assigned in two groups. Rats of group I (n=6) served as control and received only tap water and those of group II (n=6) received sodium fluoride (NaF, MW 41.99, 99% pure, Qualigens Chemicals, Mumbai, India) @ 200 mg/L of drinking water ad lib for a period of 90 days. The dose of NaF to induce toxicity was selected based on published literature and earlier studies conducted in our laboratory (Miu, et al., 2002; Yan, et al., 2007; Ranjan, et al., 2009; Dey, et al., 2011).

After 90 days, the animals were deprived of food overnight and sacrificed by decapitation. Incisor teeth were harvested and dentin was quickly removed using a scalpel. The dentin was snap frozen in liquid nitrogen and stored at -80°C for total RNA extraction.

RT and real-time PCR for collagen: Dentin stored at -80°C was transferred to liquid nitrogen and crushed to become powder. The powdered material was transferred to 1.0 ml of TRIzol reagent and homogenized (Life Technologies, USA) to isolate the total RNA as per Yan et al. (2007). RT was performed using the first strand cDNA synthesis kit (Fermentas, Life Sciences) in a volume of 20 µl containing 5 µg of total RNA, 0.5 µg of oligo dT primer, 20 units of RiboLock™ Ribonuclease inhibitor, 10 mM dNTP mix, 40 units of M-MuLV Reverse transcriptase in 5X Reverse transcriptase buffer. The reaction mixture was incubated at 37°C for one hour. The cDNA synthesis was confirmed by amplifying the Gapdh amplicon in PCR. Quantitative real-time PCR assay was performed with Brilliant® SYBR® Green Master Mix (Stratagene, USA) and Mx3000P spectrofluormetric thermal cycler operated by MxPro™ QPCR software. Aliquots of RT reactions were subjected to PCR in 20 µl reactions with SYBR® Green Master Mix using primers for Collal and Gapdh (Table 1). No template control was put for either gene quantification for checking the contamination in the reaction components other than the cDNA. Ten nanograms of non-reverse transcribed RNA of each sample were used as template instead of cDNA, the failure in amplification of which indicated that the cDNA samples were free from DNA. The thermal cycling conditions included an initial denaturation at 95°C for 10 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 sec for 40 cycles, fluorescence was recorded at the end point of each cycle and, dissociation (melting) curve consisting of 95°C for 30 s, followed by 55°C for 30 s and gradual increment from 55°C to 95°C at 2°C per min and lastly 95°C for 30 sec. Relative quantification of quantitative real time PCR product was performed using the comparative \( \Delta \Delta C_T \) method (Pfaffl, 2001). The results of Real time PCR were depicted as fold change of Col1al mRNA level in dentin of fluoride exposed rats with healthy rats.

RESULTS AND DISCUSSION
Total cellular RNA was quantified using Nanodrop (USA) and the RNA sample had \( A_{260/280} \) ratio between 1.8 and 2.0. Finally, gel electrophoresis with 1.5% agarose was used to detect the RNA. Initially RT-PCR for amplification of Col1al and Gapdh fragments (136 and140 bp respectively) using real time PCR primer pairs was optimized. With the respective primer concentration at 10 pmol/µl, annealing at 55°C yielded only the specific products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Primer locations</th>
<th>Product (bp)</th>
<th>Genbank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col1al</td>
<td>5′-CTTCGTGTAACCTCCCTCCATCC-3′ (sense)</td>
<td>4454-4599</td>
<td>136</td>
<td>NM_053304</td>
</tr>
<tr>
<td></td>
<td>5′-AAGTCCATGTGAAATTGTCCCA-3′ (antisense)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5′-ACATCTCACCAGCGCATG-3′ (sense)</td>
<td>684-823</td>
<td>140</td>
<td>NM_017008.3</td>
</tr>
<tr>
<td></td>
<td>5′-TTTCCAGGCGGCATG-3′ (antisense)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 1: Primer sequences with their corresponding PCR product size and position
of Gapdh and Col1a1 without a primer dimer. These conditions of amplification were used in Real time PCR for quantification of mRNA.

The expression level of Col1a1 gene in the F exposed rats is presented as Fig 1. The result showed that on day 90, the expression level of Col1a1 gene in the F exposed rats decreased by 51% compared to the healthy rats.

Excessive ingestion of fluoride results in a disorder of the teeth known as dental fluorosis. Teeth with fluorosis have an increase in porosity in the subsurface enamel (hypomineralization) because of fluoride-induced impairment in the clearance of proteins (amelogenins) from the developing teeth.

Collagens are the most abundant proteins in the mammalian body and it is well known that collagen is a target damaged by excessive F intoxication (Susheela and Mukerjee, 1981; Susheela and Sharma, 1982). The type I collagen gene, which is main structure-stabilizing component of bones and teeth, encodes two \( \alpha_1(I) \) polypeptide chains that are assembled into a collagen molecule (Kirsch et al., 2000; Semevolos et al., 2001). A number of reports indicate that high dosage of F can cause structural changes in collagen fibers and directly damage the quantity/quality of the collagen of the connective tissues (Pu et al., 1996; Wardas et al., 2002).

The present study showed that the F intoxication in rats down-regulates the expression level of the Col1a1 gene in dentin by 51% on day 90th of the experiment as compared with the healthy control. Earlier experiments have also demonstrated significant negative effects of fluoride on type I collagen in the ribs of rabbits, and the teeth of sheep and guinea pigs (Li et al., 2007; Yan et al., 2007; Han et al., 2010; Wang et al., 2010).

In conclusion, this study demonstrated that F can decrease the gene expression levels of Col1a1 in the dentin of incisor teeth of rats. The results of present study may thus help elucidate the complex mechanisms involved in dental fluorosis.

REFERENCES


