DECREASED EXPRESSION OF TYPE I COLLAGEN AND DENTIN PHOSPHOPROTEIN IN TEETH OF FLUOROSED SHEEP

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SUMMARY: To investigate effects of fluoride (F) on type I collagen (COL1A1) and dentin phosphoprotein (DPP) in animal dentition, teeth from sheep affected by industrial fluorosis were collected for undertaking haematoxylin and eosin (HE), Masson, and immunohistochemical (IHC) staining, plus using real-time fluorescence quantitative reverse transcription polymerase chain reaction (QRT-PCR) for assessing the expression levels of COL1A1 and DPP. These levels were reduced significantly in the dentin of both the incisor and molar teeth of the fluorosed sheep. From these findings we propose that DPP is another target for the action of F on teeth.

Keywords: Dentin phosphoprotein; Fluorosed sheep; Sheep teeth; Type I collagen in dentin.

INTRODUCTION

Previous investigations demonstrated that bones and teeth are the primary sites of fluoride (F) accumulation in humans and animals with chronic fluorosis.[1-3] Interestingly, epidemiological studies indicate that the ratio of dental fluorosis to skeletal fluorosis is near 20 to 1 in high F areas.[4,5] These findings provided two important clues for the present study: (1) teeth are more sensitive to excessive F intake than bones; (2) the mechanisms underling F-induced lesions in teeth and bones lesions may be different.

In mature dentin, collagenous proteins such as type I collagen (COL1A1) and type II collagen (COL2A1) and non-collagenous proteins (NCPs) make up approximately 20% of the organic components. NCPs play crucial roles in the mineralization of the dentin matrix and the process of forming tertiary dentin.[6] Dentin phosphoprotein (DPP), the main component of NCPs, is the key factor in mineralization.[6,7] The mutation of DPP has been shown to induce dystrophy of dentin and deafness,[8,9] and a similar symptom occurs in animals lacking COL1A1, thus suggesting that COL1A1 and DPP may participate jointly in some manner in the process of mineralization.

In the present study, with the help of real-time fluorescence quantitative reverse transcription polymerase chain reaction (QRT-PCR) and immunohistochemistry (IHC), we quantified the mRNA and protein expression levels of COL2A1 and DPP in the dentin of teeth of sheep with dental fluorosis caused by industrial F pollution.

MATERIALS AND METHODS

Experimental animals: Four three-year-old female Inner Mongolian small-tail sheep with mottled enamel from an industrial F polluted region and another four

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sheep of the same breed were chosen as controls from a non-polluted low-F region of Baotou, China. The second pair of lower incisors and molars were collected and washed three times with normal saline solution. The right incisors and molars were quickly stored in liquid nitrogen for gene expression tests, while the left ones were fixed with 4% polyoxymethylene for 24 hr, decalcified with 10% EDTA for 4 weeks, dehydrated, and embedded in paraffin for haematoxylin and eosin (HE) and Masson staining, and IHC studies.

Staining by HE and Masson: After embedding in paraffin, the teeth were serially sectioned to widths of 5 μm and stained with HE. The appropriate dyeing time for HE staining was determined to obtain the best conditions for visualizing the distinctive dental structure. The procedures of Masson were followed according to the protocol recommended for the kit (Baso Diagnostic, Inc., Zhuhai, China).

Total RNA extraction and QRT-PCR: After the teeth were pulverized in liquid nitrogen, the total cellular RNA was extracted and studied by the method of Chen et al.[10] According to the alignments of the published mRNA sequences of COL1A1 and β-actin genes in sheep and DPP genes in humans, three pairs of specific primers (Table 1) were designed by Primer 3 Plus software. The primers of the COL1A1 gene and the DPP gene were designed to amplify a 138 base pair (bp) and a 101 bp transcript, respectively. The endogenous house-keeping gene β-actin was used as a control to normalize the quantity of COL1A1 and DPP transcripts with its primers designed to amplify a 100 bp transcript.

Table 1. Primer sequences with their corresponding PCR product size and position

Gene	Primers	Primer Locations	Product Base-pairs	Genebank accession No.
ß-actin	CCAGCACGATGAAGATCAAG ATCTGCTGGAAGGTGGACAG	1051-1150	100	NM_001009784
COL1A1	AAGACATCCCACCAGTCACC TAAGTTCGTCGCAGATCACG	77-214	138	AF129287
DPP	GCCACAAACAGAAGCAACAC TCTTGGACAACAGCGACATC	562-662	101	NM_014208

The expression levels of the COL1A1 and DPP genes were quantified by the method of Li et al.[11] The QRT-PCR protocol included reverse transcription at 42ºC for 5 min and an initial denaturation at 95°C for 10 sec. This was followed by 40 PCR cycles consisting of a denaturation step at 95°C for 5 sec, an annealing step at 60°C for 20 sec, and an extension step at 72°C for 6 sec. Finally, the melting curve analysis was performed at 95°C for 15 sec, at 60°C for 1 min, and at 95°C for 15 sec as in the protocol for the three reaction steps. The amplified products were analyzed by agarose gel electrophoresis.

Immunohistochemistry: The procedures were processed according to the protocol recommended for the COL1A1 and DPP immunohistochemistry kit. After deparaffinization and rehydration, sections were incubated separately using goat anti-COL I polyclonal antibody (1:100, Santa Cruz Biotechnology, Inc.) for 3 hr at 37°C and rabbit anti-DPP polyclonal antibody (1:50, Uscn Sciences Co. LTD., Wuhan, China) for 2 hr at 37°C, and washed 3 times in PBS. Next, biotin-labeled anti-goat secondary antibody (Haoyang Biological Manufacture Co. LTD. Tianjin, China) and biotin-labeled anti-rabbit secondary antibody (Uscn Sciences Co. LTD., Wuhan, China) were introduced and the mixture was incubated for 20 min at 37°C. The specificity of the antibodies was tested by omission of the primary antibodies. After washing in PBS, tissues were visualized with DAB (diaminobenzidine) and counterstained with haematoxylin. After infusion in xylene, the sections were mounted under coverslips for microscopic examination.

Statistical analysis: An independent sample t-test (Statistical Package for the Social Sciences, SPSS 11.0) was performed to analyze differences in COL1A1 and DPP protein expression levels between fluorosed and control group. Differences with $p<0.05$ were considered statistically significance.

RESULTS

HE and Masson staining: Collagen fiber in control dentin showed neat rows (Figure 1: A, C) whereas in fluorosed dentin collagen fiber showed loose and crooked rows, non-uniform dyeing, even forming obviously holes damage (Figure 1: B, D).

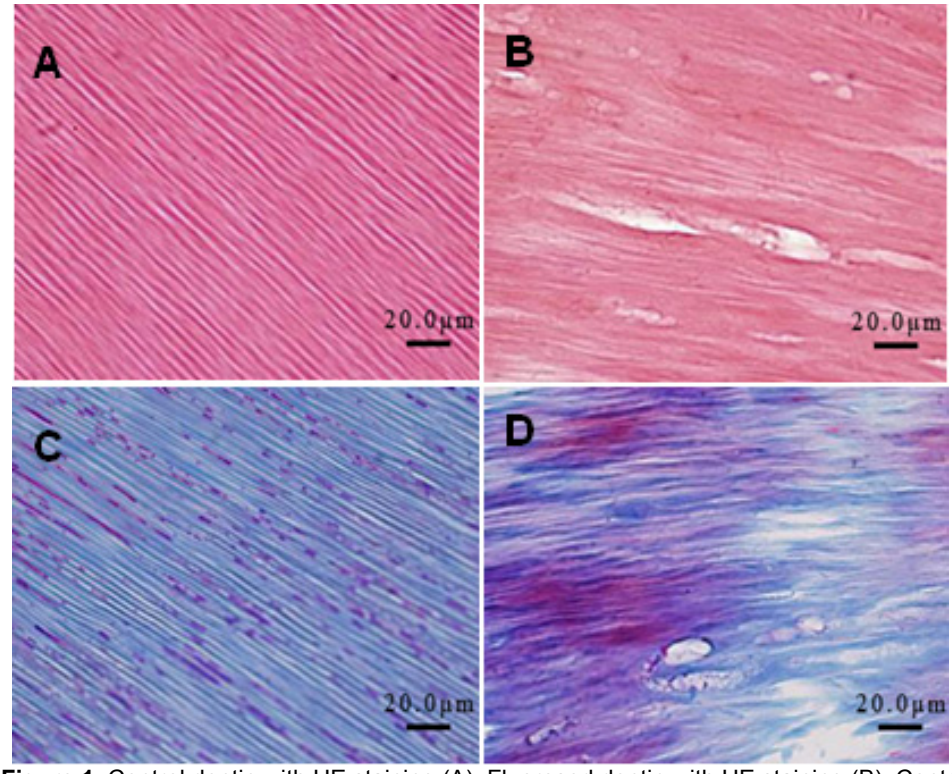

Figure 1. Control dentin with HE staining (A). Fluorosed dentin with HE staining (B). Control dentin of Masson (C). F fluorosed dentin of Masson (D). In C and D, the collagen and cartilage show up in blue and celluloses in red.

COL1A1 and DPP gene expression: The relative expression levels of COL1A1 and DPP mRNA in incisor and molar teeth of the sheep are shown in Figure 2.

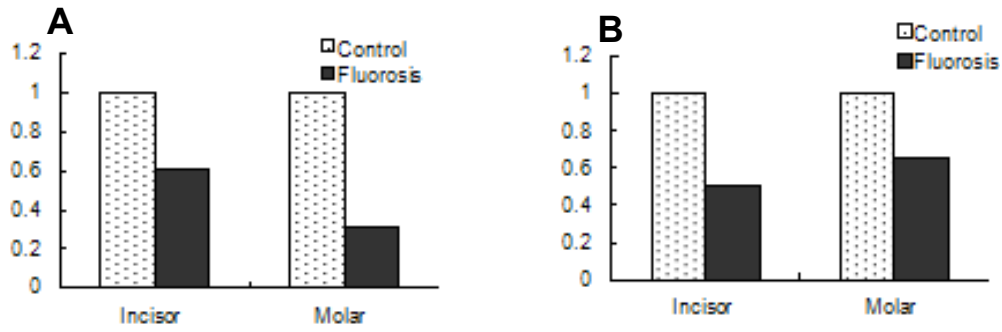

Figure 2. Relative expression level of COL1A1 gene in dentin of incisor and molar teeth (A). Relative expression level of DPP gene in dentin of incisor and molar teeth (B).

In comparison with the control group, the expression level of the COL1A1 gene in the fluorosis group was reduced in the dentin by 39.6% in the incisor teeth and 69.8% in the molar teeth, respectively. Compared with the control group, the expression level of the DPP gene in the fluorosis group was reduced by 50% in the incisors and 34.3% in the molars, respectively.

Immunohistochemistry for COL1A1 and DPP: The COL1A1 and DPP protein expression levels by immunohistochemical detection in the dentin of incisor and molar teeth from the sheep are recorded in Table 2.

Table 2. Expression of COL1A1 and DPP measured as optical density in dentin of sheep (mean± sem, n=4)

Group	Incisor COL1A1	Molar COL1A1	Incisor DPP	Molar DPP
Control	22.12±0.56	32.93±0.56	23.66±1.09	24.40±1.05
Fluorosis	18.68±0.89*	22.11±1.03*	17.08±0.98*	20.76±1.11*

*p<0.05 Fluorosis group compared with Control group, respectively.

Compared with the control group, the expression levels of COL1A1 and DPP protein in the fluorosis group were reduced in the dentin of the molar and incisor teeth, respectively.

DISCUSSION

Since the end of the 1960s, industrial F pollution has been reported in Baotou, Inner Mongolia.[1,11-16] Previously, we studied clinical manifestations, histological changes, and amino acid contents of fluorosed teeth and bone of goats in polluted areas of this province.[12-15] In this study, we chose sheep from a polluted area in Baotou to investigate the expression of COL1A1 and DPP in their fluorosed teeth.

It is well known that collagen is a target damaged by excessive F.[2,3] Our previous work has reported that F can disrupt collagen structure, calcium deposition, tooth mineralization, and dentin conformation.[12] According to earlier studies from our laboratory and other research,[7,8,17] we speculate that F may act on other crucial proteins involved in tooth mineralization besides collagen.

DPP, secreted from dentinoblasts, comprises 50% of NCPs. It is located in tropocollagen with highly repeated Asp-Ser-Ser, which can combine with Ca^{2+}. As the hydroxyapatite crystal nucleus,[6] DPP participates in tooth mineralization, through impacting on the interaction between epithelial-mesenchymal compounds, promoting the directional differential growth of odontoblast, adjusting crystal habit and growth velocity, and resisting proteolytic enzymes to ensure the calcification of the dentin matrix.[6,18-20] Therefore, in addition to COL1A1, we determined the expression of DPP in teeth of sheep with fluorosis. Results showed that the protein and gene expression levels of COL1A1 and DPP were reduced in the dentin of the fluorosed incisor and molar teeth. The findings were well supported by an earlier investigation in which F affected the phosphorylation of DPP by changing its structure to disturb the calcification of dentin.[21] In addition, Wang et al.[13] found that hydroxyproline (the main amino acid of collagen), aspartic acid, and serine (the main amino acids of DPP), were significantly reduced in dental fluorosis, providing indirect evidence for the possibility of low DPP protein level.

In summary, this study demonstrated that F can decrease the gene and protein expression levels of COL1A1 and DPP in the dentin of incisor and molar teeth of sheep. The results of this work may thus help elucidate one of the mechanisms by which F influences tooth mineralization.

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REFERENCES

1 Wang JD, Zhan CW, Chen YF, Li JX, Wang WF, Cai JP. A study of damage to hard tissue of goats due to industrial fluoride pollution. Fluoride 1992;25(3):123-8.
2 Susheela AK, Sharma YD. Certain facets of F^- action on collagen protein in osseous and nonosseous tissues. Fluoride 1982;15(4):177-90.
3 Pu CJ, Hou LH, Yang SH. Effects of excessive fluoride on metabolism of collagen protein in cartilage matrix. Chin J Control Endem Dis 1996;11(2):75-7. [in Chinese].
4 Wang HZ, Shen EL, Hou PS. The progress of prevention and control of local fluorosis in China. ISFR XX conference memoirs. Beijing, China: 1994. p. 23-5.
5 Li GS. Pathogenesis of local fluorosis. Beijing: Science Press; 2004.
6 Xie QF. Dental anatomy and oral physiology. Beijing: Peking University Medical Press; 2005.
7 Luo SJ, Li YJ. Study of DPP inducing mineralization. Chin J Stomatol 2003;38(1):56-8. [in Chinese].
8 Rajpar MH, Koch MJ, Davies RM, Mellody KT, Kielty CM, Dixon MJ. Mutation of the signal peptide region of the bicistronic gene DSPP affects translocation to the endoplasmic

reticulum and results in defective dentine biomineralization. Hum Mol Genet 2002;11(21): 2559-65.

9 Sreenath T, Thyagarajan T, Hall B, Longenecker G, D'Souza R, Hong S, et al. Dentin sialophosphoprotein knockout mouse teeth display widened predentin zone and develop defective dentin mineralization similar to human dentinogenesis imperfecta type III. J Biol Chem 2003, 278(27): 24874-80.

10 Chen HC, Hong JH, Niu RY, Sun ZL, Wang JD. Influence of F on caspase-3 expression in kidney of rats fed a normal or a low Ca and protein diet and its reversal by protein and calcium supplementation. Fluoride 2009;42(2): 121-6.

11 Li WT, Yang LF, Zhou BH, Yan XY, Wang JD. Effect of industrial fluoride pollution on COL2A1 gene expression in rib cartilage of Inner Mongolia cashmere goats. Fluoride 2006, 39(4): 285-92.

12 Wang JD, Hong JH, Li JP, Guo YH, Zhang JF, Hao JH. Effect of high fluoride and low protein on tooth matrix development in goats. Fluoride 2002;35(1):51-5.

13 Wang JD, Guo YH, Liang ZX, Hao JH. Amino acid composition and histopathology of goat teeth in an industrial fluoride pollution area. Fluoride 2003;36(3):177-84.

14 Wang JD, Hong JH, Li JX, Cai JP. The effect of nutrition supplementation during the annual dry grass season on tooth wear in industry-fluorosed goats. Fluoride 1994;27(3):136-40.

15 Wang JD, Hong JP, Li JX. Studies on alleviation of industrial fluorosis in Baotou goats. Fluoride 1995;28(3):131-4.

16 Li WT, Yang LF, Ren YC, Yan XY, Wang JD. Quantification of rib COL1A2 gene expression in healthy and fluorosed Inner Mongolia cashmere goats. Fluoride 2007;40(1):13-8.

17 Yan X, Feng C, Chen Q, Li W, Wang H, Lv L, et al. Effects of sodium fluoride treatment in vitro on cell proliferation, apoptosis and caspase-3 and caspase-9 mRNA expression by neonatal rat osteoblasts. Arch Toxicol 2009;83:451-8.

18 Xu P, Guo X. Present research on effect of fluoride acting on collagen metabolism. Foreign Med. Sci. (Section of Medgeography) 1998,19(4):152-7. [in Chinese].

19 Hao J, Zou B, Narayanan K, George A. Differential expression patterns of the dentin matrix proteins during mineralized tissue formation. Bone 2004;34(6): 921-32.

20 Hao YQ, Wang GQ, Niu ZY. Effects of fluoride on dentin. Chin J Conserv Dent 2003,13(2):112-4. [in Chinese].

21 MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, Gu TT. Dentin phosphoprotein and dentin sialoprotein are cleavage products expressed from a single transcript coded by a gene on human chromosome 4. Dentin phosphoprotein DNA sequence determination. J Biol Chem 1997, 272(2): 835-42.

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