EFFECTS OF FLUORIDE AND CHITOSAN ON THE GENE EXPRESSIONS OF BONE MORPHOGENIC PROTEIN 2 AND COLLAGEN TYPE-1 ALPHA 1 CHAIN IN THE MOUSE FEMUR

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ABSTRACT: To study the harmful effect of fluoride (F) and the alleviating role of chitosan on the development of skeletal fluorosis, we focused on serum alkaline phosphatase (ALP) and serum tartrate-resistant acid phosphatase (StrACP) activities, and the gene expression levels of collagen type-1 alpha 1 chain (COL1A1) and bone morphogenic protein 2 (BMP-2), which are fundamental to osteoblast commitment and differentiation. Sixty-eight healthy, 28-day-old, Kunming male mice were randomly divided into 4 groups and administrated sodium fluoride (NaF, 45 mg/L) and/or chitosan (5%) in their drinking water and diet, respectively. After 100 days, compared to the control group, the ALP activity was significantly ($p<0.01$) increased in the F-exposed group, while the StrACP activity was decreased ($p<0.05$). Real-time PCR was used to quantify the gene expression levels of BMP-2 and COL1A1. In contrast to the controls, the gene expressions of both BMP-2 and COL1A1 were significantly reduced by exposure to F ($p<0.05$). The results in the F+chitosan group showed that chitosan could weaken the gene inhibition of BMP-2 and COL1A1 induced by the excessive F. The findings suggest that F changed the gene related to bone formation. In turn, chitosan alleviated, to a certain extent, the F-induced damage.

Keywords: BMP-2; Bone formation; Chitosan; COL1A1; Femur; Skeletal fluorosis

INTRODUCTION

Fluoride (F), the ion of the highly reactive element fluorine, is naturally present in the environment, at varying concentrations.[1] It is clear that F is retained in the bones of humans and animals. Further, F can promote the proliferation of osteoblasts, the bone-forming cells, at low concentrations, although bone strength is not increased[2,3] and the long-term intake of excessive F may lead to F toxicity, including skeletal and dental fluorosis.[4-6] Fluorosis is a prevalent endemic disease worldwide, especially in China, one of countries in which F causes serious harm.[7]

The prevention and cure of F toxicity is extremely difficult and, for the last half-century, researchers have been exploring the mechanism of fluorosis and searching for drugs to antagonize it. On the one hand, a variety of approaches have suggested that understanding the effect of F on subcellular signaling mechanisms is of paramount importance.[8] Bone formation and destruction require a series of key signals such alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (StrACP), collagen type-1 alpha 1 chain (COL1A1) and bone morphogenic proteins (BMPs), as well as wingless proteins (Wnt). On the other hand, wide public concern about the effects of fluoride poisoning on the body has led to

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treatments for alleviating bone pain, and increasing bone density, and research looking for fluorosis antagonists.[9,10]

Chitosan is a natural polycationic linear polysaccharide obtained by partial deacetylation of insoluble naturally occurring chitin.[11] With excellent properties of biocompatibility, non-toxicity, and low allergenicity, chitosan has been used for in a wide variety of applications.[12] In our previous study, our findings suggested that chitosan may suppress F-induced damage by regulating the expression of the molecules involved in the Wnt signaling.[13] BMP-2 genes are closely related to Wnt-mediated signals and play an important role in bone formation (Figure 1). Collagen is regarded as an important treatment target in skeletal fluorosis as changes occur in the condition in both skeletal mineral metabolism and collagen, and in the serum activities of ALP and StrACP. In addition, both ALP and StrACP are sensitive to inhibition by clinically relevant concentrations of fluoride.

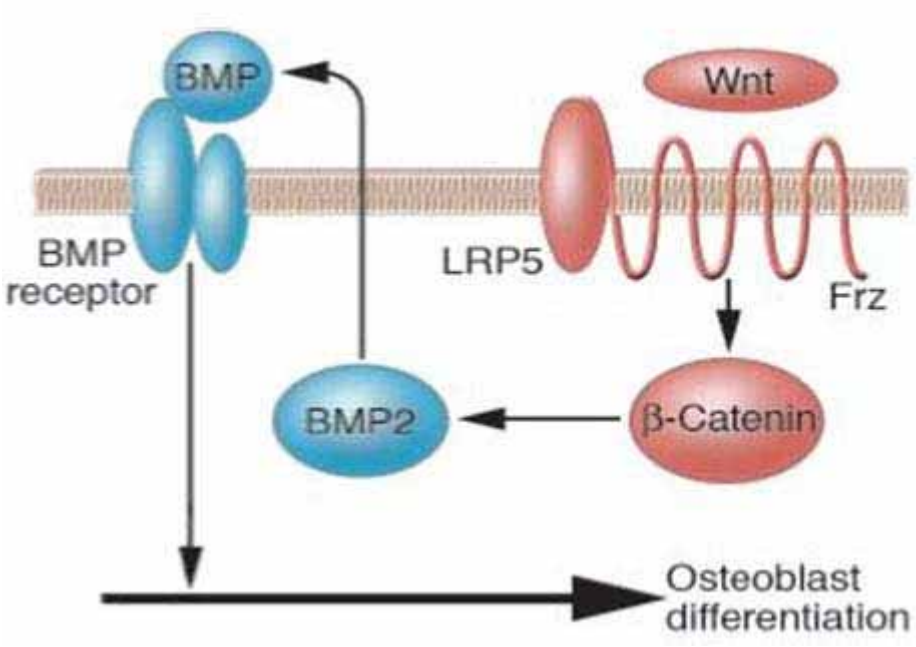

Figure 1. The relationship between the BMP signaling pathway and the Wnt signaling pathway. BMP: bone morphogenic protein, Wnt: wingless protein.

We have not had a clear understanding of the mechanism by which the combination of F and chitosan act on BMP-2 and COL1A1 genes and of the effects of this interplay. Therefore, in the present study, bone metabolism and turnover were studied by observing the activities of the serum ALP and StrACP. In addition, in order to further elucidate the influence of chitosan in fluorosis, the effect of chitosan on the bone changes in F toxicity in mice were examined by measuring the expression levels of the BMP-2 and COL1A1 genes,

MATERIALS AND METHODS

*Animals and treatment***:** Sixty-eight healthy Kunming male mice, weighing 18±2 g, supplied by the Experimental Animal Center of Shanxi Medical University, were randomly divided into 4 groups of 17 each: (i) control group (distilled water and normal diet); (ii) F-exposed group (distilled water with 45 mg F^-/L and normal diet); (iii) F+chitosan group (distilled water with 45 mg F^-/L and diet with 5% chitosan); and (iv) a chitosan-treated group (distilled water and diet with 5% chitosan). All mice were maintained on normal standard diets under standard temperature (22–25ºC), ventilation, and hygienic conditions. The animals were kept for 100 days in order to assure that they were successfully exposed to F.[14]

All protocols were approved by the Institutional Animal Care and Use Committee of China. The mice and the powdered feed were provided by the Experimental Animal Center of Shanxi Medical University of China.

Femur and serum collection: After 100 days, the general condition of mice was observed. Blood was sampled from the eyeball in the mice which were sacrificed by exsanguination with enucleation. The blood was centrifuged at 2,500 revolutions/min for 10 min to separate the serum and stored at –80ºC. At the same time, the enterocoelia of the mice were quickly opened and the femurs removed and cleaned of soft tissues. The femurs were snap frozen in liquid N_2 and stored at –80ºC to use for Western blotting,

Measurements of ALP and StrACP: The enzymatic activity of ALP and StrACP from serum in each group was measured using spectrophotometry, as described in the respective specifications in the kits.

Measurements of BMP-2 and COL1A1 gene expressions: The effect of F and/or chitosan exposure on the level of BMP-2 and COL1A1 gene expressions in the femur was analyzed by total RNA extraction and QRT-PCR. The total RNA was extracted from the femur using the Trizol reagent and the method given in the manufacturer's protocol. It was then reverse-transcribed using a PrimeScript® RT Master Mix. RT-PCR (real-time fluorescence quantitative reverse transcription polymerase chain reaction) was performed using the Mx3000P™ QRT-PCR system (Stratagene, USA). According to the alignments of the published mRNA sequences of BMP-2 and COL1α1 and β-actin genes of guinea pigs in the National Center for Biotechnology Information (NCBI), three pairs of specific primers were designed with Primer 5.0 software and synthesized with material from Biological Technology Co of Beijing (Table 1).

The reaction conditions for the first step were 37ºC for 15 min and 85ºC for 5 sec. The reaction conditions for the second step were as follows: after initial denaturation at 95ºC for 15 sec, 50 PCR cycles were started with thermocycling conditions at 61ºC for 15 sec, 72ºC for 6 sec, 95ºC for 60 sec, 55ºC for 30 sec, and then 95ºC for 30 sec, followed by the reaction melting curve analysis to verify the specificity of the amplified products.

Statistical analysis: The data were expressed as mean±SD. T-tests, performed with GraphPad Prism 5 software, were used to analyze the gene expression levels of BMP-2 and COL1A1. The significance level for all the tests was set at $p<0.05$.

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

Gene	Primer sequences	Accession no.	Product sizes (bp)
β-actin	F: GATCATTGCTCCTCCTGAGC R: ACATCTGCTGGAAGGTGGAC	NM_007393	83
COL1A1	F: TGACTGGAAGAGCGGAGAGT R: GTTCGGGCTGATGTACCAGT	NM_007742	151
BMP-2	F: CTGCAGCAAGAACAAAGCAG R: CCCTGGAAGGGATTATAGGC	NM_007553	110

RESULTS

The general condition of the mice: Over the whole experimental period, the mice in the control group were in good health, with thick and glossy hair on their bodies, responded rapidly to stimuli, and had a full complement of teeth. In contrast, the mice in the F-exposed group were disheveled, had a light covering of matte, rather than glossy, hair, and dental fluorosis. Compared with the control group, the physical condition of the mice in the F+chitosan group was a little worse while the condition of those in the chitosan-treated group was not significantly different from that of the control group.

Measurements of ALP and StrACP: Compared to the control group, the enzymatic activity of ALP tended to increase in each of the experimental groups with the difference in the F-exposed group being significant ($p<0.01$, Figure 2).

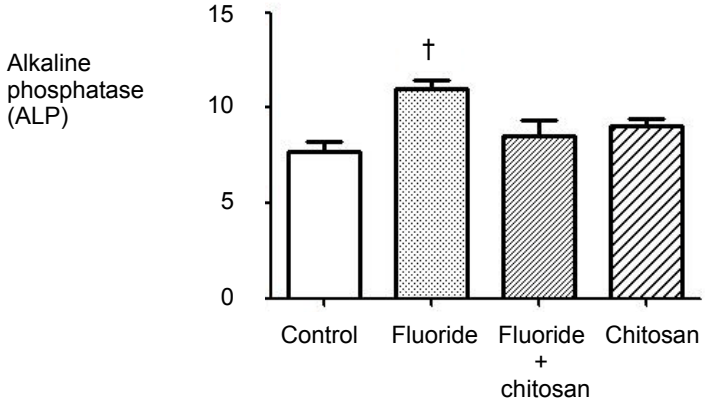

Figure 2. Serum alkaline phosphatase (ALP) activity (U/100mL) in the groups (n=8; mean±SE). Compared to control: †$p<0.01$

The enzymatic activity of StrACP was significantly reduced in the F-exposed group compared to both the control and the F+chitosan groups ($p<0.05$, Figure 3).

Effects on BMP-2 and COL1A1 gene expression: In the F-group, compared with the control group, the mRNA levels of both BMP-2 and COL1A1 in the mice femurs were significantly decreased ($p<0.05$, Figure 4). In contrast, no significant differences were present between the F+chitosan group and the control group.

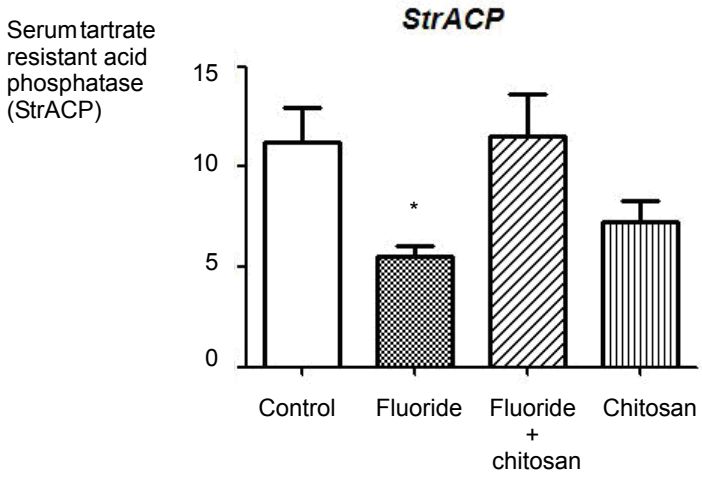

Figure 3. Serum tartrate resistant acid phosphatase (StrACP) activity (U/100mL) in the groups (n=8; mean±SE). Compared to the control and to the fluoride+chitosan groups: *p<0.05

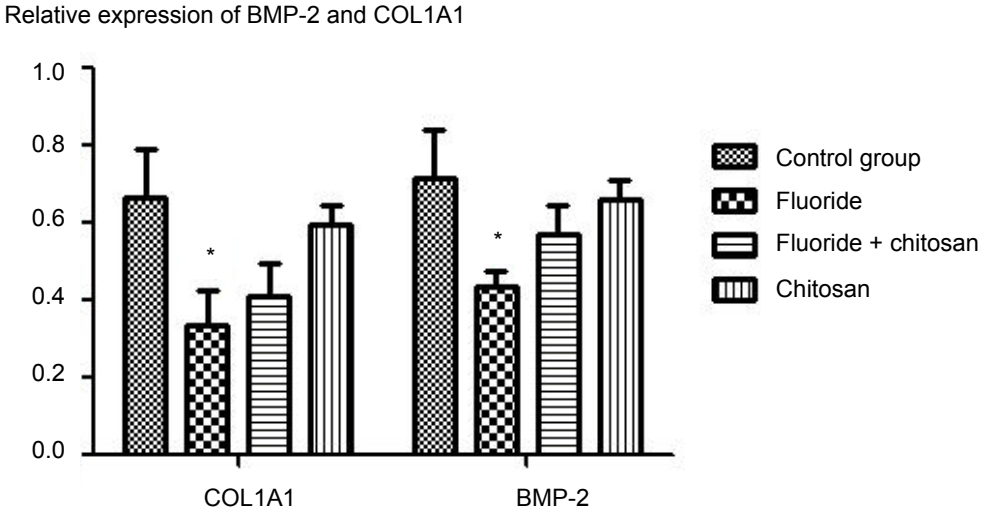

Figure 4. Effects of chitosan and fluoride on the related gene expression of BMP-2 and COL1A1 (n=4; mean±SEM). Compared to control: *p<0.05

DISCUSSION

ALP, of which approximately 50% comes from osteoblasts, has been considered as an early and crucial indicator in the evaluation of bone formation and metabolism.[15,16] F can affect ALP activity through changing the enzyme structure and influencing the cell vitality of the bone.[17] In skeletal fluorosis, the changes in the serum ALP may be related to an active proliferation of the osteoblasts. In this experiment, as shown in Figure 2, the ALP activity in the F-exposed group was significantly elevated. This suggests that a stimulation of bone metabolism occurs in fluorosis.[18] The absence of a difference in the ALP activity in the F+chitosan group, compared to the control group, suggests that chitosan can alleviate the damage caused by fluoride.

StrACP exists mainly in osteoclasts and the lack of it may lead to osteosclerosis.[19] In the process of bone resorption, osteoclastic activity is high and the osteoclasts simultaneously secrete a substantial amount of StrACP. Therefore, StrACP is often regarded as an important indicator of bone resorption. *In vitro*, the formation of osteoclasts is reflected in increases in the StrACP concentration.[20] In addition, there is evidence that StrACP is also expressed in bone forming cells (osteoblasts and osteocytes) and is mediated by F.[21] This research has confirmed that changes in StrACP activity can mirror the effect of F on bone transformation and repair. In the present study, a significant decrease in StrACP was observed when the F-exposed group was compared to both the control and the F+chitosan groups, suggesting F exposure may slow bone transformation and repair (Figure 3).

In contrast to the significant changes in ALP and StrACP in the F-exposed group, compared to the control group, no significant changes occurred in these parameters in the F+chitosan group suggesting that chitosan may be able to antagonize, in part, the development of fluorosis (Figures 2 and 3).

An interesting phenomenon has been discovered in Adélie penguins (*Pygoscelis adeliae*) where, despite a bone F concentration as high as 9000 μg/g, no clinical symptoms of skeletal fluorosis are present.[22] Yin et al. suggested that in the krill in the Adélie penguins' diet, which contain abundant chitin, might both prevent the absorption of some of the dietary inorganic fluoride and also stabilize the absorbed F as organically bound fluoride, mainly in the form of fluorinated chitin.[22] Therefore, how chitin affects the action of F in bone cells has become a research hotspot. Chitin, coming from the exoskeleton of insects, crustaceans (mainly shrimps and crabs), and the cell walls of fungi, is also the second most abundant natural polysaccharide after cellulose.[23,24] Many reports have found that chitosan has a strong ability to bind cations. Xia et al. found that chitosan can promote the absorption of calcium by making Ca^{2+} soluble in the intestines through chelating Ca^{2+}, thus preventing the accumulation of CaF_2 in the skeleton and enhancing the release of free fluoride ions.[25]

What is noteworthy is that chitosan may also promote bone formation. and has played a major role in bone tissue engineering.[26-30] Chitosan has been reported to accelerate bone regeneration in rat tibia and has been used for bone repair in cases

of dental extraction.[31] During the process of bone formation and remodeling, bone morphogenetic proteins (BMPs) and collagen are currently considered to be the key components that control the growth and differentiation of bone cells.

BMP-2 is a member of the multi-functional transforming growth factor β (TGF-β) superfamily and can promote osteoblastic proliferation, differentiation, and attachment.[32] *In vivo* studies have demonstrated that BMP-2 also promotes osteoblastic activity via increasing ALP activity and calcium mineral deposition.[33-36] BMP-2 may induce ectopic bone formation and is a necessary substance for osteanaphysis.[37,38] Collagen type-1 fibers are formed from collagen protein and provide a matrix for bone calcium deposition. The synthesis of collagen type-1 is enhanced by BMP-2.[39] Deletions of COL1A1 have been demonstrated in osteogenesis imperfecta type I.[40] These studies indicate that not only BMP-2 but also COL1A1 plays an important role in bone formation. Therefore, we chose these two genes to inquire into the mechanism of fluorosis and the joint effects of F and chitosan on skeleton. In the present experiment, when compared to the control group, the expressions of BMP-2 and COL1A1 in the F-exposed group were obviously decreased. In addition, the addition of chitosan partly alleviated these adverse effects. Therefore, we hypothesize that chitosan is able to activate the formation of BMP-2 and COL1A1 which in turn inhibit the damage caused by F. Thus chitosan can partly alleviate the F-induced differential gene expressions of BMP-2 and COL1A1 in the femurs of mice.

CONCLUSION

The present study examined the role of chitosan in skeletal formation in F-exposed mice by measuring the changes in serum ALP and StrACP and the expression levels of the BMP-2 and COL1A1 genes. Our findings suggest that the activities of ALP and StrACP in bone metabolism and transformation can be changed by F. In addition we found that chitosan, to a certain extent, may ameliorate F-induced damage by regulating the gene expressions of BMP-2 and COL1A1, both of which are involved in osteogenesis.

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