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Effect of fluoride on the expression of type I collagen gene (COL1A1) in skeletal muscle of rats

Shashi A*, Nidhi Rana

Department of Zoology and Environmental Sciences, Punjabi University, Patiala-147002, Punjab, India.

E-mail : shashiuniindia@yahoo.co.in Contact No. : +91-8054280827

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Abstract

The present study assessed the effect of high fluoride ingestion on expression levels of Type I Collagen gene (COL1A1) in skeletal muscle of rats. Thirty Wistar albino rats of both sexes were randomly assigned to three groups. The group I served as control and received 1ml double distilled water/kg/bodyweight/day orally for 40 days. The groups II and III were treated with 300 and 600 ppm of sodium fluoride in drinking respectively for the same period. The animals were sacrificed under ether anesthesia , skeletal muscle was excised and used for RT-PCR assay. Total RNA was isolated by homogenization in TRIzol, followed by the single step purification method . The RNA concentration was estimated using a spectrophotometer and an equal amount of total RNA was reverse-transcribed to synthesize single-stranded cDNA. Total cellular RNA was quantified using Nanodrop (USA). The RNA sample had A260/280 ratio between 1.78 and 1.88. Finally, gel electrophoresis with 1.5% agarose gel was used to detect the RNA. When the skeletal muscle mRNA was used for cDNA synthesis, a 136-bp product was produced using specific COL1A1 PCR primers. Controls lacking reverse transcriptase resulted in the production of no detectable PCR product. The expression level of collagen gene in skeletal muscle of fluoride exposed rats decreased as compared to the controls. Thus, investigating the effect of fluoride on the expression levels of COL1A1 of skeletal muscle provide basic data for further elucidating the molecular mechanisms of skeletal damage induced by fluoride toxicity.

Key words : COL1A1 gene , Fluoride , Polymerase Chain Reaction , Rat skeletal muscle.

INTRODUCTION

Endemic fluorosis is now known to be a global issue, and understanding the pathogenesis of the disease marks for scientific investigation. The skeletal muscle exhibits very high level of plasticity and undergoes atrophy or wasting in a variety of disease states [1] including fluorosis [2, 3].

Collagen is a major component of bone and cartilage and is present in all connective tissue that require strength and flexibility [4]. Skeletal muscle tissue contains four main collagen types *viz ;* I, III , IV and V . Type I and Type III collagens constitute the major fibrillar collagens, with fibrils containing a core of Type V collagen. Type I collagen is present primarily in the epimysium and perimysium of muscle fiber [5] . The extracellular matrix of skeletal muscle is collagen rich tissues that are important for muscle function [6].

Type I collagen , the main collagen type expressed in bone [7] consists of two α 1 (I) and one α 2 (I) polypeptide chains that assemble into functional collagen protein. The α 1 (I) and α 2 (I) subunits are encoded as COL1A1 and COL1A2, respectively, and have different amino acid sequences [8,9]. Many experimental studies have shown that fluoride can cause structural changes in collagen fibers and directly damage the quantity/quality of the collagen of the connective tissues [10].

The present study was designed to assess the effect of high fluoride ingestion on expression levels of Type I Collagen gene (COL1A1) in skeletal muscle of rats, providing basic data for further elucidating the molecular mechanisms of skeletal damage induced by fluoride toxicity.

MATERIALS AND METHODS

Experimental design

Thirty Wistar albino rats weighing 150-200 g were provided with standard rodent chow (Hindustan lever limited,Mumbai, India) and water was supplied *ad libitum*. After a 15 days acclimatisation period, they were randomly divided into 3 groups of ten animals each. The rats of group 1 served as control and received double distilled water one ml/ kg bw/day via oral gavage for 40 days. Group II and III were given sodium fluoride (NaF, 99 % pure , Loba chemie, Mumbai, India) in the doses of 300 and 600 mg /kg bw/day in their drinking water for the same period respectively.

Ethical Aspects

The conduct and procedures involving animal experiments were approved by the Ethics Committe on Animal Experiments of Punjabi University, Patiala, India.

Tissue preparation

At the end of 40 days, the experimental animals of 3 groups were sacrificed under ether anaesthesia, and the skeletal muscle tissues were collected . Aliquots of muscle samples were kept in RNA *later* ™ (Ambion , USA) for isolation of RNA and stored at 20° C.

Total RNA extraction and analysis

Muscle samples stored in RNA *later* ™ were homogenised in 0.5 ml TRIzol reagent (Life Technologies, USA), and total RNA was isolated as per manufacturer' s instructions. The RNA extracts were treated with RNase free DNase I to remove contaminating DNA, quantified on a spectrophotometer and stored at 80° C. An equal amount of total RNA (5µg per reaction)

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

Gene	Primers	Primer Locations	Product (bp)	Genbank accession No .
Col1A 1	5'-CTTCGTGTAAACTCCCTCCATCC-3' (sense) 5'-AAGTCCATGTGAAATTGTCTCCCA-3' (antisense)	4454- 4599	136	NM_ 053304

was reverse transcribed to synthesize single- stranded cDNA using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit # K1621.

PCR amplification

PCR amplification was performed using the following conditions

2 µL of single stranded cDNA from a reverse transcription reaction was amplified at 95° C for 5 minutes for one cycle followed by 98° C for 20 seconds, 72° C for 30 minutes, 72° C for 4 minutes for 35 cycles.

RT-PCR products were loaded and run on 1.5% agarose gels. Gels were stained with ethidium bromide in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0), visualized under UV transilluminator and photographed. Images were scanned and saved as tagged image files (TIF s) using Adobe Photoshop™ Software (Adobe Systems Inc., Mountain View, CA) .

RESULT

Total cellular RNA was quantified using Nanodrop (USA) and RNA sample had $A_{260/280}$ ratio between 1.78 to 1.88.

The skeletal mRNA used for cDNA synthesis produced a 136-bp PCR product using COL1A1 specific PCR primer. Controls lacking reverse transcriptase resulted in the production of no detectable PCR product. RT-PCR using COL1A1 PCR primers resulted in visible amplified products with skeletal muscle mRNA (Fig. 1).

A single band of the predicted size (136 bp) was obtained using specific primers for skeletal muscle. The COL1A1 gene expression was more expressed in Group III (600mg NaF) than Group II (300mg NaF) which indicated that COL1A1 gene was more affected by fluoride in Group III as compared to Group II (Fig.2).

DISCUSSION

Although many studies have shown that fluoride can cause structural changes in collagen fibers, little is known about the molecular mechanisms of these effects.

Collagen is the most abundant extracellular matrix protein in mammals. Twenty-seven types of collagen have been reported [11], Collagen types I and IV are major components of the connective tissues and basal lamina, respectively [12].

Type I collagen is a heterotrimer protein consisting of two α 1 (I) and one α 2 (I) polypeptide chains . The two polypeptides are synthesized in a 2 : 1 stoichemistry. The same 2 : 1 ratio is observed for the corresponding mRNA . The Collagen I gene is encoded as COL1A1 and COL1A2 genes [13].

The present study revealed that expression of Type I Collagen gene in skeletal muscles was decreased in fluoride treated rats. The findings are consistent to the previous experiments that demonstrated significant fluoride induced disturbances of Type I Collagen gene expression in ribs of rabbits [14], teeth of guinea pigs [15] and fluorosed sheep [16], osteoblasts [13] and dentin [17] of rats.

In the present study, the RNA preparations contained no contaminating DNA, since all RT- negative controls showed no detectable amplifications. Our findings showed that administration of 600 mg NaF /kg/bw/day in drinking water inhibited the expression of COL1A1 mRNA in the skeletal muscle of rat, and the depression effects were also detected in 300 mg NaF. The findings are also in agreement with the study of Wang *et al*. [15] which reported that administration of 150 mg NaF / L in the drinking water inhibited the expression of COL1A1 gene in the teeth of guinea pigs.

Another report indicates that expression of COL1A1 significantly decreases when administered a dose of 0.5 mg/L and

Table 2: Quantitative estimation of skeletal muscle Total RNA

Sample	A_{260nm}	A_{280nm}	$A_{260/280nm}$	RNA Quantity(µg/ µl)	RNA Yield(µg)
L1(CONTROL)	0.108	0.060	1.8	0.432	21.6
L2(300)	0.073	0.041	1.78	0.292	14.6
L3(600)	0.047	0.025	1.88	0.188	9.4

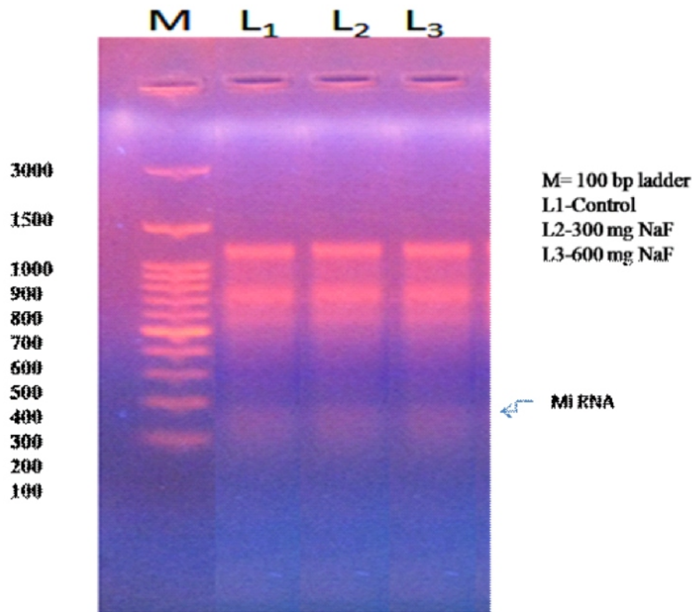

Figure 1: Determination of the optimal concentration of skeletal muscle total RNA required for reverse transcriptase-polymerase chain reaction (RT-PCR) assay of COL1A1 mRNA concentrations.
The RT-PCR reaction was carried out as described in material and methods using 25ng of total RNA .
M - (100 bp ladder) containing molecular weight markers.
Lane 1 - Group I (Control)
Lane 2 - Group II (300mg NaF)
Lane 3 - Group III (600mg NaF)

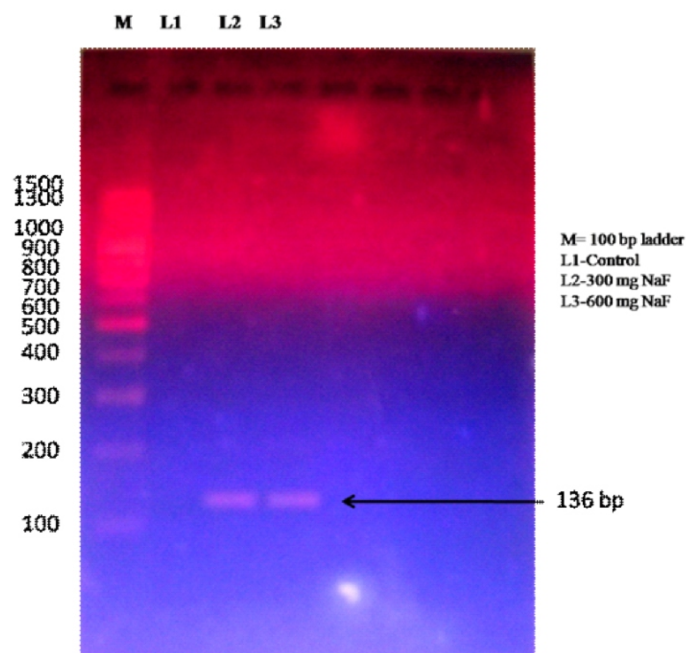

Figure 1: Ethidium bromidestained agarose gel showing results of PCR amplification.
M - (100 bp ladder) containing molecular weight markers.
Lane 1 - Group I (Control)
Lane 2 - Group II (300mg NaF)
Lane 3 - Group III (600mg NaF)

1 mg/L of fluoride for 48 hours [8]. However , some workers have suggested that expression of COL1A1 mRNA increased in osteoblasts after exposure to fluoride for 48 and 72 hours [18].

CONCLUSION

In conclusion, this study demonstrated that fluoride decreased gene expression levels of COL1A1 mRNA in the skeletal muscle of rats. The results of this investigation may help elucidate for better understanding of molecular mechanisms of fluoride toxicity and play an important role in etiology of skeletal fluorosis.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

REFERENCES

1. Jackman RW, Kandarian SC .The molecular basis of skeletal muscle atrophy. Am. J. Physiol. Cell Physiol. 2004: 287:834-843.

2. Shashi A. Fluoride toxicity and muscular manifestations: histopathological effects in rabbit. *Fluoride* 1989: 22(2): 72-77.

3. Shashi A , Sharma N. The pathology of muscle leisons in experimental fluorosis. *Int. J. Sci. Innovations Discoveries* 2011 : 1(2): 255-262.

4. Prockop , D J , Kivirikko K I . Collagens: molecular biology, diseases and potentials for therapy *Ann. Rev. Biochem*. 1995 : 64: 403-434.

5. Best TM, Shehadeh SE, Leverson G, Michel JT, Corr DT, Aeschlimann D. Analysis of changes in mRNA levels of myoblast- and fibroblast derived gene products in healing skeletal muscle using quantitative reverse transcription-polymerase chain reaction. J. Orthop. Res. 2001: 19 : 565-572.

6. Light, Champion AE. Characterization of muscle epimysium, perimysium and endomysium collagens. *Biochem. J.* 1984: 219: 1017-1026.

7. Nicolai M, Michael H, Cvrilla M, Rainer H. Expression of type I and type II in conscecutive stages of human osteoarthritis. Histochem. Cell Biol. 2004:122 (3) : 229-237.

8. Miu Q , Xu M , Liu Bc, You BR, Kang N. In vivo and in vitro study on the effect of excessive fluoride on type I collagen of rats. J. of Hygiene Research 2002: 31(3) : 145-147.

9. Xu Z L , Liu BC , Miu Q. Effects of excessive fluoride on the expression type I collagen mRNA and fiber characterstics in rat bone. J. Environ. Health 2003: 20 (4) : 204-207.

10. Shashi A, Bhushan B, Bhardwaj M. Histochemical pattern of gastrocnemius muscles in fluoride toxicity syndrome. *Asian Pacific J. Trop.Med*. 2010: 3(2): 136-140.

11. Myllyharju J, Kivirikko KI. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet. 2004 : 20 : 33-43.

12. Timpl R, Widemann H, Van Delden V , Furthmayr H , Kuhn K . A network model for the organization of type IV collagen molecules in basement membranes. Eur. J. Biochem. 1981 : 120 : 203-211.

13. Yan XY, Li WT, Zhou BH, Wang JM. Effect of supplemented protein and calcium nutrition on fluoride induced disturbances of rib COL1A1 gene expression in rabbits. *Fluoride*

2007 : 40:140-148.

14. Han TL , Wang M , Yan XY , Niu RY , Wang TD. Decreased expression of type 1 collagen and dentin phosphoprotein in teeth of fluorosed sheep. *Fluoride* 2010: 43:19-24.

15. Wang M, Han TL, Cao CF, Wang JD. Effects of aluminum and fluoride on the expression of type 1 collagen in the teeth of guinea pigs. *Fluoride* 2010: 43: 45-51.

16. Yan XY, Yan XT, Morrision A, Han TL, Chen QL, Li J, Wang JD. Fluoride induces apoptosis and alters collagen I expression in rat osteoblasts. *Toxicol. Letrs*. 2011 : 200 : 133-138.

17. Gupta AR, Dey S, Saini M, Swarup D. Down regulation of expression of type 1 collagen gene in dentin of fluoride exposed rats. Indian J. Anim. Res. 2014 : 48 : 601-604.

18. Li D, Liang YH , Fan Z, Li GS. Influence of fluoride on type I mRNA and protein expression in rat osteoblasts : an in vitro study. Chin. J. Endemiol. 2006: 25 (2) : 129-131.