EFFECTS OF EXCESSIVE FLUORIDE INGESTION ON COLLAGEN PROTEIN AND EXPRESSION OF TYPE I COLLAGEN GENE IN SKELETAL MUSCLES OF RATS

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SUMMARY: The aim of this study was to ascertain the effect of excessive ingestion of F on the metabolism of type I collagen in skeletal muscles of rats. Eight-week-old female rats (n = 36) were divided into two equal groups. Rats of group I served as control and received only tap water while those of group II received 200 mg NaF/L in their drinking water *ad libitum* for a period of 90 days. At the end of the experiment, the group II rats showed significantly higher concentrations of F in plasma, bone, urine, and faeces. On the other hand, the level of hydroxyproline in skeletal muscles of these F-exposed rats (452.10±22.54 µg/gm) was significantly lower than in the group I control rats (817.51±32.06 µg/gm). Acid- and pepsin-soluble collagen concentrations of skeletal muscles, however, were increased significantly ($p < 0.05$) in the group II rats (10.42±0.57 and 70.33±2.37 µg/mg) compared to the group I control rats (6.48±0.38 and 41.99±1.25 µg/mg), respectively). The expression level of type I collagen (COL1a1) gene in the F-exposed rats decreased by 54% as compared to the control rats. In conclusion, the study found that excessive F ingestion in rats leads to increased degradation and solubility of the collagen protein along with down-regulation of the expression of the COL1A1 gene in the skeletal muscles.

Keywords: COL1A1 gene expression; Collagen metabolism; Fluoride and collagen; Hydroxyproline; Rat muscles; Real-time polymerase chain reaction (PCR); Skeletal muscles.

INTRODUCTION

Although both dental and skeletal fluorosis have been studied extensively, more information is desirable on the effects of fluoride (F) on skeletal muscles, since they contain relatively high levels of calcium susceptible to the uptake of F ions. As a major component of mammalian muscle tissue, collagen is present in all connective tissues that require strength and flexibility.[1] Susheela[2-4] was one of the first to observe that collagen protein is damaged by excessive F ingestion. She and her colleagues demonstrated experimentally with animals and from human studies that fluorosis is directly involved in skeletal muscles. They documented that toxic levels of F degenerate the actin and myosin filaments in skeletal muscle (gastrocnemius muscle) resulting in atrophy of the myofibrils. With her colleagues, her investigations confirm that excessive intake of F inhibits the synthesis of collagen, leading to its breakdown in bone, tendon, muscle, skin, cartilage, lung, kidney, and trachea.[4-8] F disrupts the synthesis by cells responsible for laying down collagen and causes these cells to try to compensate for their inability to assemble intact collagen, thereby producing larger quantities of imperfect collagen and/or noncollagenous protein. In recent

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years, a number of reports have demonstrated significant negative effects of F on type I collagen (COL1A1) in the ribs of rabbits and in the teeth of sheep, guinea pigs, and goats.[9-12]

The present study was carried out to assess the effects of excessive F ingestion by rats on collagen metabolism by determining the level of hydroxyproline, collagen solubility, and expression of the COL1A1 gene in their skeletal muscles.

MATERIALS AND METHODS

Animals and experimental design: Female Wistar albino rats (n=36), 8 weeks of age, averaging 100 g/bw, were procured from the Laboratory Animal Resource Section of the Indian Veterinary Research Institute after obtaining approval from the Institute Animal Ethics Committee (IAEC, IVRI) for the experiments. The rats were housed in polypropylene cages with a 12-hr dark/12-hr light cycle in the laboratory animal facility at a temperature ranging from 18 to 25ºC and a relative humidity of 55–60%. Throughout the experiment, the rats were provided *ad libitum* access to tap water containing 0.23 ppm F and laboratory ration consisting of 12% wheat bran, 87% maize, 1% mineral salt, and 4.20 ppm F.

After 15 days of acclimatization, the rats they were randomly assigned into two groups of uniform weight. Rats of group I (n=18) served as the control and received only the 0.23-mg F/L tap water and those of group II (n=18) received sodium fluoride (NaF, 99% pure, Qualigens Chemicals, Mumbai, India) with 200 mg NaF/L in their drinking water *ad libitum* for 90 days. The dose of NaF to induce toxicity was selected on the basis of published literature and earlier studies conducted in our laboratory.[10,13-15]

Just before the rats were sacrificed, urine samples were collected in Eppendorf tubes for F estimation. Individual fresh faecal samples were also collected on small plastic petri dishes, dried, and then kept in separate polypacks for F estimation. After overnight fasting, six rats of each group were sacrificed by decapitation on days 30, 60, and 90 of the experiment. Blood samples were collected by cardiac puncture using heparinised sterile syringes. The samples were immediately centrifuged at 3000 rpm for 20 min to separate plasma, which was used for estimation of F. Skeletal (gastrocnemius) muscles and long bones (femur and tibia) were excised and kept at –20ºC until analysed. Weighed muscle samples were placed into labelled, resealable, plastic envelopes and frozen for estimation of collagen solubility. Aliquots of muscle samples were kept in RNA*later*™ (Ambion, USA) for isolation of RNA and stored at –20ºC.

Fluoride determinations: The concentration of F in plasma and urine was estimated by the method of Cernik, et al.[16] and in faeces by the method of Madhavan and Subramanian[17] using a digital ion-analyzer equipped with a F specific electrode (Orion Research Model 290A, Cambridge, Massachusetts, USA) attached to a potentiometer (Thermo Electron Corporation, USA). F concentration in bone was estimated after ashing and using the same digital ion-analyzer.[18] Calibration was made using five freshly prepared working standards. The results are expressed in parts per million (ppm). The accuracy and precisions

of the measurements were maintained by repeated analysis of reference standard procured from Orion Research Inc. Laboratory, USA.

Quantification of skeletal muscles hydroxyproline content: Protein hydrolysates were prepared by adding 6N HCl to the muscle tissue samples in a sealed tube and heated in a heating block at 120ºC overnight. The hydrolysates were neutralized with 6N NaOH and analysed for hydroxyproline as an indirect measure of tissue collagen content in the manner described by Neuman and Logan.[19]

Solubility studies: Stored frozen muscle samples were thawed in the plastic envelope in a refrigerator. They were then diced into to small cubes, using a sharp scalpel and placed in Eppendorf tubes along with 0.5M acetic acid containing Protease Inhibitor Cocktail (Sigma) and mixed overnight on a mechanical shaker at 4ºC. The acetic acid-soluble collagen was separated by centrifugation at 15000 × g for 45 min. The residue was subjected to further digestion with pepsin (1 mg of pepsin/10 mg of tissue) in 0.5M acetic acid for a further 24 hr at room temperature. The pepsin-solubilized collagens were separated by centrifugation at 15000 × g for 45 min. The acid and pepsin soluble collagen was quantified with Sircol Collagen Assay (Bicolor, Newtownabbey, Northern Ireland) following the manufacturer's instructions.

Reverse transcriptase (RT) and real-time PCR for collagen: Muscle samples stored in RNA*later*™ were homogenized in 0.5 mL of TRIzol reagent (Life Technologies, USA), and total RNA was isolated as per manufacturer's instructions. RT was performed using the first strand cDNA synthesis kit (Fermentas, Maryland, USA) in a volume of 20 µL containing 5 µg total RNA, 0.5 µg of oligo dT primer, 20 units of RiboLock™ Ribonuclease inhibitor, 10 mM dNTP mix, and 40 units of M-MuLV Reverse transcriptase in 5X Reverse transcriptase buffer. The reaction mixture was incubated for 1 hr at 37ºC. The cDNA synthesis was then confirmed by amplifying the Gapdh amplicon in the PCR (polymerase chain reaction). Quantitative real-time PCR assay was performed with Brilliant SYBR Green Master Mix (Stratagene, USA) and Mx3000P spectrofluorimetric thermal cycler operated by MxPro™ QPCR software. Aliquots of RT reactions were subjected to PCR in 20-µL volume reactions with SYBR Green QPCR Master Mix using primers for COL1A1 and Gapdh (Table 1). Other than the cDNA, no template control was used for either gene quantification or for checking the contamination in the reaction components. 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 95ºC for 10 min, denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 45 sec for 40 cycles. Fluorescence was recorded at the end point of each cycle with the dissociation (melting) curve consisting of 95ºC for 30 sec, followed by 55ºC for 30 sec and gradual increment from 55ºC to 95ºC at 2ºC per min and lastly 95ºC for 30 sec. Relative quantification of quantitative real time PCR product was performed using the comparative $\Delta\Delta C_T$ method.[20] The results of Real time PCR were depicted as fold

change of COL1A1 mRNA level in muscle samples of F exposed rats compared with healthy rats.

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

Gene	Primers	Primer locations	Product (bp)	Genbank accession No.
Col1A1	5'-CTTCGTGTAAACTCCCTCCATCC-3' (sense) 5'-AAGTCCATGTGAAATTGTCTCCCA-3' (antisense)	4454-4599	136	NM_053304
Gapdh	5'-ACATCATCCCTGCATCCACT-3' (sense) 5'-TTTCTCCAGGCGGCATGTCA-3' (antisense)	684-823	140	NM_017008.3

Statistical analysis: Data were analyzed by one-way analysis of variance (ANOVA) with post-hoc analysis by Duncan's multiple comparison tests using SPSS 11.5 software. Results are expressed as mean±SE with $p < 0.05$ considered statistically significant.

RESULTS

Fluoride concentrations: The concentrations of F in plasma, urine, faeces, and bones of the group II F-treated rats at different observation periods are presented in Table 2.

Table 2. Concentration of F in plasma, urine, faeces, and bones in rats
(Values are expressed as mean ±S.E.; n = 6 rats; group I = control; group II = F-treated rats)

Fluoride concentration	Group	Day 30	Day 60	Day 90
Plasma (µg/mL)	I	0.094±0.014	0.104±0.019	0.111±0.005
	II	0.328±0.027[a]	0.429±0.015[*a]	0.665±0.017[†a]
Urine (µg/mL)	I	2.19±0.32	2.24±0.91	2.39±0.23
	II	46.18±0.60[a]	70.95±3.78[*a]	80.15±2.79[†a]
Faeces (µg/gm)	I	6.91±0.20	7.72±0.35	7.93±0.15
	II	29.59±2.75[a]	58.12±2.70[*a]	93.63±4.64[†a]
Femur (µg/gm)	I	980.56±68.46	979.67±23.29	984.09±44.69
	II	6259.48±283.83[a]	8387.13±482.40[*a]	9221.70±202.83[†a]
Tibia (µg/gm)	I	1062.91±35.92	1060.28±49.28	1076.18±53.06
	II	5611.06±432.66[a]	8565.89±689.70[*a]	9240.90±406.16[*a]

[a]$p < 0.05$ compared with respective control rats on different observation periods.
[*]$p < 0.05$ compared with day 30 value.
[†]$p < 0.05$ compared with day 30 and 60 values.

A significant increase in these F concentration occurred from day 30 onwards in the experimental as compared to the healthy control (group I) rats.

Hydroxyproline and collagen solubility in skeletal muscles: Mean hydroxyproline concentration (µg/g) in skeletal muscles is given in Table 3.

Table 3. Hydroxyproline concentrations and solubility of collagen in skeletal muscles of control (group I) and F-treated rats (group II); all values are expressed as mean ±S.E.; n = 6 rats.

Group	Hydroxyproline (µg/gm)	Acid soluble collagen (µg/mg)	Pepsin soluble collagen (µg/mg)
I	817.51±32.06	6.48±0.38	41.99±1.25
II	452.10±22.54*	10.42±0.57*	70.33±2.37*

*$p < 0.05$ compared with respective control rats.

Significant reduction in hydroxyproline concentration in the muscle samples of the F-exposed rats was observed at the end of the 90-day experiment.

The solubility of collagen in skeletal muscles was measured using Sircol assay (Table 3). Acid- and pepsin-soluble collagen concentrations in skeletal muscles increased significantly in the F-exposed rats by the end of the 90-day experiment.

Relative quantification of collagen mRNA expression in muscle samples: The expression level of COL1A1 gene in the F-exposed rats is presented in Table 4.

Table 4. Fold change of COL1A1 gene expression level in F-treated rats (group II) relative to healthy control (group I)

Group	ΔC_T (Avg COL1A1 C_T - Avg Gapdh C_T)	$\Delta\Delta C_T$ (ΔC_T Gr II - ΔC_T Gr I)	Fold difference in COL1A1 relative to Group I ($2^{-\Delta\Delta CT}$)
I	2.15±0.10	0±0.10	1
II	3.28±0.27	1.13±0.27	0.46

The results showed that on day 90, the expression level of this gene in the F exposed rats decreased by 54% compared to the healthy rats.

DISCUSSION

Excessive exposure to F for a prolonged period can induce chronic fluorosis. In this study, oral exposure of rats to F in their drinking water for three months resulted in significant elevation of F concentrations in plasma, urine, faeces, and bones as compared to the control group.

Collagens are the most abundant type of protein in the mammalian body, and it is well known that collagen is a target damaged by excessive F intoxication.[2-13] Many experimental studies have shown that F can cause structural changes in collagen fibers and directly damage the quantity/quality of the collagen of the connective tissues. Collagen synthesized and laid down during F exposure is under hydroxylated and inadequately cross-linked. As a consequence, this collagen is rapidly catabolized and the collagen content of the tissues is decreased.[5-8] In the

present study, the collagen levels in skeletal muscles of rats after excessive ingestion of F were calculated from the hydroxyproline concentration assuming that hydroxyproline constitutes 12.5% of collagen.[21] At the end of the experiment the hydroxyproline concentrations in the skeletal muscles of F exposed rats were decreased significantly by 44.70% ($p < 0.05$). This decrease in skeletal muscle collagen is in agreement with results of earlier studies,[7,22-25] which have demonstrated that F interferes with the collagen biosynthesis resulting in decreased collagen content. Therefore, the decrease in collagen content of skeletal muscles of rats after exposure of sodium F may be either due to decreased synthesis or increased degradation by collagenase.[7,8,26]

The amounts of acid- and pepsin-soluble collagen in the skeletal muscles of the rats were increased after exposure to F by 60.80% and 67.49%, respectively, compared to the control rats. These results therefore support previous studies,[5,24,27] indicating that lysine and hydroxylysine residues are reduced after ingestion of F, thereby producing inadequately cross-linked and increasingly acid-soluble collagen protein.

A number of reports also indicate that a high dosage of F affects the collagen metabolism of cartilage and bone[23,28,29] and leads to a decrease of type I collagen gene expression.[10-13,30] The present study reports on the expression of type I collagen gene in skeletal muscles of rats after exposure of F. The extracellular matrix (ECM) of skeletal muscle is collagen-rich tissues that are important for muscle function. The fibrillar collagens, type I and III, are the major components of muscle ECM.[31] The present study showed that the F intoxication in rats down-regulates the expression level of the COL1A1 gene in skeletal muscle by 54% on day 90 of the experiment as compared with the healthy control.

In conclusion, this study demonstrated that F exposure affects collagen metabolism and leads to increased degradation (in terms of hydroxyproline) and solubility of collagen protein along with down-regulation of the expression of the COL1A1 gene in skeletal muscles of rats.

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