TAMARIND (*TAMARINDUS INDICA*) FRUIT PULP SUPPLEMENTATION PREVENTS COLLAGEN DEGRADATION AND DOWN REGULATION OF COLLAGEN 1 GENE EXPRESSION IN FLUORIDE-EXPOSED RATS

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SUMMARY: The present investigation was undertaken to evaluate the ameliorative effect of tamarind (*Tamarindus indica*) fruit pulp extract on fluoride (F)-induced collagen degradation by evaluating the concentration of hydroxyproline, the soluble collagen pool, and the expression level of collagen I gene in skeletal muscles of Wistar albino rats. In the F-exposed rats, the level of hydroxyproline, as well as the cross-linking of the collagen, was decreased as shown by an increase in the acid and pepsin soluble collagen in the skeletal muscles. The expression of the collagen 1 gene in the F-exposed rats decreased by 54% as compared to the normal rats. The administration of tamarind fruit pulp extract by gavage for 90 days with fluoridated water increased the level of hydroxyproline and decreased the solubility of the collagen protein along with an increased expression of the collagen 1 gene in the skeletal muscles of the rats. From this study, it can be concluded that the tamarind fruit extract had a positive influence on the content of collagen and its characteristics in the F-exposed rats, along with an ability to reduce the body F burden.

Keywords: Amelioration; Collagen degradation; Fluoride; Muscles; Rats; Tamarind fruits extract (TFE).

INTRODUCTION

Collagens, the most abundant proteins in the body, constitute a multigene family of extracellular matrix proteins. Fluoride ions (F) disrupt the collagen synthesis in the cells responsible for laying down collagen leading to these cells trying to compensate for their inability to put out intact collagen by producing larger quantities of imperfect collagen and/or noncollagenous protein. Earlier experiments have revealed that F exposure inhibits the synthesis of collagen and leads to the breakdown of collagen in bone, tendon, muscle, skin, cartilage, lung, kidney, and trachea.[1-3] In recent years, a number of reports have demonstrated significant negative effects of F on the collagen 1 gene in different tissues of experimental animals.[2,4,5]

Prolonged excessive F exposure plays an important role in the development of dental, skeletal, and non-skeletal fluorosis in both humans[6-8] and domestic animals.[9-11] It is well known that osteo- and dental-fluorosis are irreversible F-generated toxic effects but their further advancement or severity can be prevented or checked by the supplementation of food nutrients.[12] A few studies have also revealed that the edible parts (fruits, flowers, leaves, pods, seeds etc.) of many Indian plant species have a vital role in the natural amelioration of fluorotoxicosis in certain species of domestic animals including goats (*Capra hircus*), sheep (*Ovis*

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aries), and camels (*Camelus dromedarus*).[13-15] One of these plants, 'tamarind', commonly known as a Imli or Ambli (*Tamarindus indica* L.), belonging to Leguminosae family, has also been shown to exert a beneficial effect on F toxicity.[16-19] However, the efficacy of tamarind on F-induced collagen degradation in skeletal muscles has never been assessed. Therefore, the present investigation was undertaken to evaluate the protective or ameliorative effect of an extract of tamarind fruit pulp on the collagen content and expression of the type 1 collagen gene in the skeletal muscles of F-intoxicated rats.

MATERIALS AND METHODS

Plant material and preparation of extract: Tamarind fruits were procured from the local market in Bareilly, Uttar Pradesh, India. The plant material was identified and authenticated from the Botanical Survey of India, Central National Herbarium, Howrah, India, (voucher No. CNH/1-1/2007 Tech11) where the voucher reference specimens were deposited. The tamarind fruits extract (TFE) was prepared as described previously.[17]

Animals and experimental design: Female Wistar albino rats (n=30) of 8 weeks of age and around 100 g body weight were procured from the Laboratory Animal Resource Section of Indian Veterinary Research Institute after obtaining approval from the Institutional Animal Ethics Committee. The rats were housed in polypropylene cages in 12 hr dark/12 hr light cycles with the temperature of the laboratory animal house ranging from 18 to 25ºC and the humidity between 55 and 60%. During the entire period of the experiment, the rats were provided with laboratory rations (composition: wheat bran: 12%, maize: 87%, salt: 1%, F: 4.20 ppm), and tap water (F: 0.23 ppm) *ad libitum*.

After a 15 day acclimatization period, they were randomly assigned to three groups of 10 rats each. The rats of group I served as a control and received only tap water; group II received 90.5 mg F/L, in the form of 200 mg sodium fluoride (NaF)/L, in their drinking water, and group III received 90.5 mg F/L, in the form of 200 mg NaF/L, in their drinking water along with a hydro-methanolic extract of tamarind fruit pulp (200 mg/kg body weight, bw) daily by gavage for a period of 90 days.

The dose of NaF to induce toxicity was selected based on the published literature and earlier studies conducted in our laboratory.[2-4] The dose of tamarind (200 mg/kg bw) was also selected based on our previous studies[17,18] in which a dose-dependent effect of tamarind on reducing the serum and bone F concentration with a concomitant increase in the excretion of F in the urine was obtained.

Sample collection and F estimation: Blood, urine, faeces, muscles, and long bone were collected and stored as described earlier.[2] The concentration of F in these biological samples was estimated with a F ion selective electrode (Orion, USA) as described previously.[2]

Quantification of skeletal muscle hydroxyproline and solubility studies: The levels of hydroxyproline and the acid and pepsin soluble collagen in the skeletal muscles were quantified as described earlier.[2]

Reverse transcriptase and real-time polymerase chain reaction (PCR) for collagen: Two pairs of specific primers (Table 1) for Col1a1 and Gapdh were designed according to the alignments of the published cDNA sequences.

Table 1. Primer sequences with their corresponding PCR product size (base pairs, bp) and position

Gene	Primers	Primer locations	Product size (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

The total RNA from the muscles was isolated as described previously.[2] Reverse transcriptase (RT) was performed using the first strand cDNA synthesis kit (Fermentas, Life Sciences) as described in a previous report.[2] Quantitative real-time PCR conditions and analysis of the Col1a1 gene expression level were the same as described earlier.[2] The results of real time PCR were depicted as the fold change of the Col1a1 mRNA level in the muscle sample of the experimental rats compared with the normal rats.

RESULTS

F concentration: The F concentrations in the plasma, urine, faeces, and bones of the normal and experimental rats are presented in Tables 2 and 3. The administration of TFE with F caused a significant reduction in the serum F level and the concentration on day 90 was 44.67% lower than in the untreated F-exposed rats. The co-administration of TFE with F showed a significant increase in urine and faecal F excretion and it was 79.06% and 20.83% higher than in the untreated F-exposed rats, respectively on day 90. A significant lower F accumulation in bones was recorded in the rats supplemented with TFE (Table 3).

Hydroxyproline and pattern of collagen solubility in skeletal muscles: The data in Table 4 depicts the level of hydroxyproline and the solubility pattern of the skeletal muscle collagen of the normal and experimental rats at the end of the experiment. The administration of TFE significantly increased the level of hydroxyproline and it was 37.10% higher than in the F-exposed untreated rats. The percentage of acid and pepsin soluble collagen was restored in the skeletal muscles of rats supplemented with TFE along with NaF.

Relative quantification of collagen mRNA expression in skeletal muscles: The expression level of the Col1a1 gene calculated by the $\Delta\Delta CT$ method in the rats of different groups is depicted in Table 5. The result showed that the decreased expression of the Col1a1 gene in the F-exposed groups increased significantly with the concomitant use of TFE.

Table 2. Concentration of fluoride in plasma, urine and faeces in rats
(Values are expressed as mean±SE; n=10 rats; group I = control;
group II = F-treated rats; group III = F + TFE)

F concentration	Group	Day 30	Day 60	Day 90
Plasma (µg/mL)	I	0.094±0.014	0.107±0.021	0.121±0.009
	II	0.341±0.035*	0.454±0.011*‡	0.685±0.029*§
	III	0.357±0.012*	0.396±0.039*	0.379±0.026†
Urine (µg/mL)	I	2.31±0.49	2.37±0.38	2.40±0.41
	II	49.93±2.69*	65.70±5.33*	74.15±3.05*‡
	III	55.73±5.38*	70.60±8.75*	132.77±16.68†§
Faeces (µg/g)	I	7.15±0.34	7.86±0.32	8.34±0.15
	II	26.50±2.08*	52.34±3.24*‡	86.04±3.02*§
	III	43.29±1.19*	69.60±4.65*‡	103.96±5.51*§

*p<0.05 compared with respective control rats on different observation periods; †p< 0.05 compared with the F-treated rats on different observation periods; ‡p<0.05 compared with the day 30 value; §p<0.05 compared with the day 30 and day 60 values.

Table 3. Concentration of fluoride in the bone of rats of different groups
(Values are expressed as mean±SE; n=10 rats; group I = control;
group II = F-treated rats; group III = F + TFE)

Group	Femur (µg/g)	Tibia (µg/g)
I	980.55±41.18	1099.80±45.87
II	9962.76±131.95*	9952.61±87.48*
III	7951.15±153.26†	8194.99±381.53†

*p<0.05 compared with the respective control rats; †p< 0.05 compared with the fluoride-treated rats.

Table 4. Hydroxyproline concentrations and solubility of the collagen in the skeletal muscles of rats (Values are expressed as mean±SE; n=10 rats; group I = control; group II = F-treated rats; group III = F + TFE)

Group	Hydroxyproline (µg/g)	Acid soluble collagen (µg/mg)	Pepsin soluble collagen (µg/mg)
I	794.08±12.50	6.81±0.22	42.89±2.07
II	439.09±36.84*	10.98±0.28*	73.00±3.79*
III	601.99±31.50†	8.58±0.14†	47.99±7.74†

*p<0.05 compared with respective control rats; †p<0.05 compared with fluoride-treated rats.

Table 5. Fold change of Col1a1 gene expression level in the F-treated rats (group II) and the F + TFE group (group III) relative to the healthy control (group I) (Data are presented as mean±SE for 10 rats in each group).

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
III	2.83±0.54	0.68±0.54	0.62

DISCUSSION

A protective effect of TFE, on the F-induced collagen degradation in the skeletal muscles of rats, was observed in the present investigation. The hydroxyproline concentration in the skeletal muscles of the F+TFE group was comparable with that of the normal rats. The decreased hydroxyproline in the tissues indicates a decrease in the biosynthesis of collagen or an increase in degradation of collagen. Both of these possibilities have been observed by various researchers in F-exposed animals.[2,3,20] The plasma and tissue levels of ascorbic acid have been reported to be decreased in experimental fluorosis.[21] Ascorbic acid is known to be a co-factor for the enzyme prolyl hydroxylase,[22] which is required for the hydroxylation of proline to form hydroxyproline. The beneficial effect of TFE on tissue collagen might be due to the presence of ascorbic acid in the extract[13] thus maintaining the activity of the prolyl hydroxylase enzyme and decreasing the rate of collagen degradation in the skeletal muscles.[23]

The percentage of acid and pepsin soluble collagen was increased in the skeletal muscles of the F-exposed rats, indicating that the collagen produced during this period is inadequately cross-linked. It is noted that a decrease in cross-links increases the solubility of the collagen.[24] In the F-exposed rats, supplementation with TFE reduced the solubility of the collagen protein. The ameliorative effect on the solubility of the collagen protein might be due to the presence of copper in TFE,[25] which helps in maintaining the cross-links of the collagen fibers. The beneficial effect of copper in reducing the level of hydroxyproline in urine has been reported in F-exposed rabbits.[26]

Collagens are the most abundant proteins in the mammalian body and it is well known that collagen is damaged in F intoxication.[1] The gene for type I collagen, the main structure-stabilizing component of the bones and teeth, encodes the two α1 (I) polypeptide chains that are assembled into a collagen molecule.[27] A number of reports indicate that a high dosage of F can cause structural changes in collagen fibers and directly damage the quantity/quality of the collagen of the connective tissues.[2-5] The present study revealed a down regulation of the expression level of the Col1a1 gene by 54% on the 90th day in the F-exposed rats as compared with the normal rats. The co-administration of TFE with F prevented the down-regulation of the expression of Col1a1 gene as compared to the F-exposed rats, indicating the beneficial effect of TFE against F toxicity. The ameliorative potential of TFE on the expression of type 1 collagen gene might be due to the presence in TFE of high concentrations of calcium and ascorbic acid.[13, 23]

The TFE significantly reduced the plasma and bone F concentrations and enhanced the excretion of F through both urine and faeces. Interference with the F absorption from the gut due to the administration of TFE might have played a role in reducing the plasma F concentration.[18] Compared to the ability of TFE to reduce the plasma F concentration, tamarind has a relatively low ability to reduce the bone F concentration. TFE may act by preventing the deposition of F in bone or by chelating out F already integrated into bones.

The increased excretion of F through urine and faeces in the F+TFE group over the positive control rats suggested that the extracts facilitated F excretion by acting at both the digestive and metabolic levels. Copper is reported to prevent F accumulation in bone.[26] A high concentration of copper has been found in TFE[25] and this may have contributed towards the reduced bone F deposition in the rats receiving TFE. Iron and zinc form insoluble complexes with fluoride ions at the gut level and interrupt its absorption.[16] The presence of these trace elements in the TFE might make an additional contribution to the reduction of body F burden with TFE.[28]

The renal clearance of F is influenced by the urinary pH and the rate of clearance is higher with an alkaline urine.[29] Tartaric acid, a major component of tamarind paste, does not get metabolized and this leads to the production of an alkaline urine which promotes urinary F excretion.[30] The increase in the pH of the urine with tamarind fruit pulp supplementation is a possible mechanism for the increased urinary F excretion with TFE.[7] These factors may be responsible for the

reduced concentration of F in bones and its enhanced excretion in rats receiving TFE.

In conclusion, the TFE had a positive influence on the content of collagen and its characteristics in the F-exposed rats, along with an ability to reduce the body F burden. Further work is currently underway studying the beneficial effects, on the collagen content in F-exposed animals, of the components of the TFE.

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