Interdiscip Toxicol. 2016; **Vol. 9**(1): 12–16.
doi: 10.1515/intox-2016-0002

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interdisciplinary toxicology

ORIGINAL ARTICLE

Toxic effect of sodium fluoride on hydroxyproline level and expression of collagen-1 gene in rat bone and its amelioration by *Tamrindus indica* L. fruit pulp extract

Amit Raj GUPTA, Sahadeb DEY, Mohini SAINI, Devendra SWARUP

Environmental Medicine Laboratory, Division of Medicine, Indian Veterinary Research Institute, Izatnagar - 243122, India

ITX090116A02 • Received: 13 October 2015 • Revised: 15 February 2016 • Accepted: 18 February 2016

ABSTRACT

Excessive fluoride intoxication plays an important role in the development of dental, skeletal and non-skeletal fluorosis. The aim of this study was to ascertain the toxic effect of excessive fluoride ingestion on the level of hydroxyproline and expression of type 1 collagen gene in rat bone and its amelioration by supplementation with *Tamarindus indica* fruit pulp extract. Forty albino rats were randomly assigned to four groups. The first group served as control and received only tap water. The second group received sodium fluoride (200 ppm) through drinking water. The third group received *T. indica* fruit pulp extract (200 mg/kg body weight) alone and the fourth group received the *T. indica* fruit pulp extract (200 mg/kg body weight) along with fluorinated drinking water (200 ppm) daily by gavage for a period of 90 days. The level of hydroxyproline and expression of type 1 collagen gene using quantitative real time PCR in the tibia bone decreased significantly with continuous exposure to sodium fluoride. Co-administration of *T. indica* fruit pulp extract during exposure to fluoride through drinking water restored the level of calcium, phosphorus and alkaline phosphatase in serum and the concentration of hydroxyproline in urine. It increased the level of hydroxyproline and expression of type 1 collagen gene in the tibia as compared to untreated fluoride-exposed rats. It is concluded that T. indica fruit pulp extract has an ameliorative potential to protect the bone from fluoride induced collagen damage.

KEY WORDS: *Tamarindus indica*; fluoride; hydroxyproline; collagen-1; rats

Introduction

Fluorosis is a common problem in livestock and human beings in endemic areas characterized by a crippling condition. The clinical entity is manifested by signs of bony exostosis, lameness, dental discoloration, painful mastication, poor performance, reduced production and death. Industrial origin of excess fluoride and its presence in the immediate environment, soil, plant and water is incriminated as one of the principal sources of continual low doses of fluorine to livestock through feed, fodder and water (Patra *et al.*, 2000).

Collagen is the major structural protein in the extracellular matrix of musculoskeletal tissues. Bone and teeth, comprising mostly type 1 collagen, are the major target organs of fluoride (Pu *et al.*, 1996). Fluoride disrupts the collagen synthesis resulting in production of imperfect collagen and/or non-collagenous protein in cells. Studies have shown that fluoride can negatively affect collagen metabolism and lead to the breakdown of collagen in different tissues of experimental animals (Yan *et al.*, 2007; Gupta *et al.*, 2013a; Gupta *et al.*, 2014; Gupta *et al.*, 2015a). In India, medicinal plants have long been used as traditional medicines for the treatment of various health problems. Earlier studies reported that medicinal herbs like tamarind fruit pulp, fruit and seeds of *Moringa oleifera*, bark extract of *Terminalia arjuna* and blackberry juice played a protective role against fluoride induced toxic effects (Sinha *et al.*, 2007; Hassan & Yousef, 2009; Ranjan *et al.*, 2009; Khandare *et al.*, 2010; Gupta *et al.*, 2015b).

Correspondence address:
Amit Raj Gupta
Teaching Veterinary Clinical Complex
College of Veterinary Science and Animal Husbandry
Orissa University of Agriculture and Technology
Bhubaneswar-751003, Odisha, India
TEL.: +919658830748 • FAX +916742391780, 2391424
E-MAIL: dramitrajgupta@gmail.com

Tamarindus indica L. (Leguminosae), commonly known as tamarind, is a tree native to Africa, also cultivated in Sudan, Indonesia, Pakistan, Philippines, Java, Spain and Mexico. In India, it is colloquially known as Imli or Indian date. Different parts of tamarind, like fruits, fruit pulp, seeds, leaves, flowers, and barks, have been used for various medicinal purposes.

In recent years, a number of reports documented the beneficial effect of tamarind in fluoride toxicity (Khandare *et al.*, 2010; Dey *et al.*, 2011; Gupta *et al.*, 2013b; Gupta *et al.*, 2013c; Gupta *et al.*, 2015c). However, the efficacy of tamarind on fluoride induced collagen degradation in bone has not yet been assessed. The present study was therefore conducted to evaluate the ameliorative effect of hydro-methanolic extract of *T. indica* fruit pulp on the hydroxyproline level and expression of type 1 collagen gene in rat bone continually exposed to 200 parts per million (ppm) of sodium fluoride in drinking water.

Materials and methods

Experimental animals

Female albino Wistar rats of eight weeks of age, weighing 150–170 g, bred in the Laboratory Animal Resource Section of IVRI, were used. The animals received normal laboratory pellet diet (composition: wheat bran – 12%, maize – 87%, salt – 1%, F – 4.20 ppm) and water (F – 0.23 ppm) *ad libitum*. The animals used in the present study were maintained in polypropylene cages at 12 h dark/12 h light cycles, with temperature of the laboratory animal house ranging from 18 to 25 °C and humidity between 55 and 60%. The study was approved by the Institute Animal Ethics Committee (Approval number: 1-53/2004-JD Research, Dated: 09.04.2010).

Plant material and preparation of extract

Tamarind fruits were purchased from the local market in Bareilly, Uttar Pradesh, India. The plant material was duly authenticated from the Central National Herbarium, Botanical Survey of India, Government of India (Botanical Garden, Howrah-103) and voucher specimen no. CNH/1-1/2007/Tech-11 was assigned to it. The extract was prepared as described previously (Gupta *et al.*, 2013c).

Experimental procedure

Forty rats were randomly divided into four groups of ten rats each. Group I served as control and received only tap water; group II received 200 ppm sodium fluoride (NaF, MW 41.99, 99% pure, Qualigens Chemicals, Mumbai, India) through drinking water. Group III received hydro-methanolic extract of *T. indica* fruit pulp (200 mg/kg body weight) alone and group IV, *T. indica* fruit extract (200 mg/kg body weight) along with fluorinated drinking water (200 ppm NaF), daily by gavage for a period of 90 days. The dose of sodium fluoride to induce toxicity was selected according to the published literature and earlier studies conducted in our laboratory (Yan *et al.*, 2007; Gupta *et al.*, 2013a; Gupta *et al.*, 2015a). The dose of *T. indica* (200 mg/kg body weight) was based on previous studies in our laboratory (Dey *et al.*, 2011; Gupta *et al.*, 2013b; Gupta *et al.*, 2013c) in which a dose-dependent effect of *T. indica* on reducing serum and bone fluoride concentration with concomitant increase in excretion of fluoride in urine was obtained.

Sample collection and evaluation of biochemical parameters

Blood, urine and tibia bone were collected and stored as described in a previous report (Gupta *et al.*, 2013a). Fluoride concentration in bone, level of hydroxyproline in bone and urine, along with serum biochemical parameters were assessed as described earlier (Gupta *et al.*, 2015a).

Reverse transcriptase (RT) and quantification of Col1a1 gene expression

Tibia bone stored at –80 °C was transferred to liquid nitrogen and crushed to powder. The powdered material was transferred to 1.0 ml of TRIzol® reagent (Life Technologies, USA) and homogenized to isolate the total RNA according to the protocol of Yan *et al.* (2007). Reverse transcriptase (RT) was performed using the first strand cDNA synthesis kit (Fermentas, Life Sciences) as described in a previous report (Gupta *et al.*, 2013a). Quantitative real-time PCR conditions and analysis of the Col1a1 gene expression level were the same as described earlier (Gupta *et al.*, 2013a) (Table 1). The results of real time PCR were depicted as fold change of Col1a1 mRNA level in the tibia bone of experimental rats compared to normal rats.

Statistical analysis

Each sample was run in duplicate. Data were analyzed by one-way analysis of variance (ANOVA), with post hoc analysis using Duncan's multiple comparison tests using SPSS 16 software, and expressed as mean ± SE (n=10) with $p<0.05$ considered statistically significant.

Results

At the end of the experiment, the accumulation of fluoride in the tibia bone was significantly high ($p<0.05$)

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

Gene	Primers	Primer locations	Product (bp)	Genebank 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

in fluoride-exposed rats as compared to controls. The concomitant use of *T. indica* fruit pulp extract along with fluoride significantly (p<0.05) reduced the fluoride accumulation in the tibia bone and the fluoride concentration was 17.66% lower than in the fluoride-exposed non-treated group. The rats that received *T. indica* fruit pulp extract alone were not significantly different from healthy controls (Table 2).

The level of hydroxyproline in tibia bone of normal and experimental rats is presented in Table 2. The hydroxyproline level in tibia bone of the control group and the group that received *T. indica* fruit pulp extract alone was found to be comparable. Hydroxyproline concentration in tibia bone of fluoride-exposed untreated rats was significantly (p<0.05) reduced as compared to healthy control rats. Administration of *T. indica* fruit pulp extract along with fluoride significantly increased the level of hydroxyproline in tibia bone of experimental rats.

Calcium and phosphorus concentrations in serum of healthy control rats and rats that received *T. indica* fruit pulp extract alone remained statistically comparable at different intervals of the experiment (Table 3). The concentration of calcium decreased significantly (p<0.05) between day 30 and 90 as compared to the healthy control group and there were also significant progressive declines in serum calcium concentrations from day 30 onwards till day 90 in the fluoride-exposed group. However, there was a significant (p<0.05) increase in the values of phosphorus concentration of fluoride-exposed rats between day 30 and day 90 compared to healthy control values. Concomitant use of *T. indica* fruit pulp extract restored the calcium and phosphorus concentration and the mean levels were comparable to the respective values in control rats.

Serum alkaline phosphatase activity at different observation periods in healthy and experimental rats is presented in Table 3. The all-day serum alkaline phosphatase activity was significantly higher in the fluoride-exposed rats than in the healthy control rats (p<0.05) and there were also significant progressive rises in serum alkaline phosphatase activity on day 30, 60 and 90 in the fluoride-exposed group (p<0.05). Administration of the *T. indica* fruit pulp extract significantly reduced serum alkaline phosphatase activity compared to the fluoride-exposed group at different observation periods.

The rats that received sodium fluoride in their drinking water revealed a significantly (p<0.05) higher concentration of hydroxyproline in urine throughout the experiment as compared to healthy control rats. Concentrations of hydroxyproline in the treatment group receiving *T. indica* fruit pulp extract were comparable with those of respective healthy control values (Table 3).

The expression level of Col1a1 gene calculated by the $\Delta\Delta C_T$ method in rats of different groups is given

Table 2. Concentration of fluoride and hydroxyproline level in tibia bone of rats.

Group	Fluoride concentration (µg/gm)	Hydroxyproline (mg/gm)
I	1099.8±45.87	25.9±0.35
II	9952.6±87.48*	20.1±0.49*
III	1088.3±35.65	25.6±0.68
IV	8194.9±381.53**	23.1±1.05**

The values are expressed as mean ± S.E. (n = 10).
*p<0.05 compared with respective control rats; **p<0.05 compared with fluoride treated rats.

Table 3. Calcium and phosphorus concentration, activity of alkaline phosphatase in serum, and level of hydroxyproline in urine of rats.

Parameter	Group	Day 30	Day 60	Day 90
Calcium (mg/dl)	I	7.0±0.28	6.9±0.26	6.9±0.13
	II	6.7±0.23*	5.6±0.10*†	4.7±0.26*‡
	III	7.0±0.21	6.9±0.23	6.9±0.18
	IV	6.9±0.29B**	6.5±0.18**	6.8±0.39**
Phosphorus (mg/dl)	I	5.5±0.34	5.6±0.31	5.5±0.05
	II	6.5±0.21*	7.1±0.25*	7.8±0.19*†
	III	5.6±0.28	5.5±0.26	5.5±0.16
	IV	5.8±0.59	6.1±0.38	5.9±0.41**
Alkaline phosphatase (units/ml)	I	23.3±0.67	24.5±0.75	23.8±0.56
	II	35.7±0.68*	45.5±0.69*†	76.3±0.82*‡
	III	23.5±0.52	23.9±0.38	25.1±0.44
	IV	26.7±0.57**	34.1±0.40**†	45.1±1.73**‡
Urinary hydroxyproline (µg/ml)	I	164.1±14.75	180.1±6.59	200.0±15.12
	II	228.9±17.22*	262.9±8.80*†	301.6±11.71*‡
	III	165.2±12.56	181.2±5.68	201.1±13.26
	IV	173.6±13.55**	197.9±17.03**†	226.4±31.64**‡

The values are expressed as mean ± S.E. (n=10).
*p<0.05 compared with respective control rats at different observation periods; **p<0.05 compared with F treated rats at different observation periods; †p<0.05 compared with day 30 value; ‡p<0.05 compared with day 30 and 60 values.

Table 4. Fold change of col1a1 gene expression level in rats of different groups relative to healthy control.

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	3.38±0.08	0±0.08	1.00
II	4.58±0.13	1.21±0.13	0.43*
III	3.34±0.07	–0.03±0.07	1.02
IV	3.81±0.11	0.49±0.11	0.71**

Data are reported as mean ± S.E. for 10 rats in each group.
*p<0.05 compared with respective control rats; **p<0.05 compared with fluoride treated rats.

in Table 4. The result showed decreased expression of Col1a1 gene in tibia bone of fluoride-exposed rats, which increased significantly with concomitant use of *T. indica* fruit pulp extract.

Discussion

Tamarind has long been used as traditional medicine for the treatment of a wide variety of ailments and diseases. Pods (fruits) are rich in ascorbic acid and sugar and have been used as spices in syrup, juice, drinks and many food preparations. Medicinally, the fruit pulp is valued as cathartic, astringent, febrifuge, antiseptic and refrigerant (Komutarin *et al.*, 2004). The extract of tamarind fruit is reported to contain a high concentration of zinc, a nutritional antioxidant (Glew *et al.*, 2005), fat, carbohydrates, fiber, ash, calcium, phosphorus, iron, magnesium, sodium, thiamine, riboflavin, niacin, vitamin C and tannin (Coronel, 1999). It also contains pectin, 2,3,5, tri-o-methyl arabinose, 2,3,4,tri-o-methyl D xylose, 2,3,4,6,tetra-o-methylgalactose, 2,3,6,tri-o-methyl D-glucose, organic acids, mainly potassium-hydrogen tartarates, further free acids such as tartaric, malic and citric acids, as well as phenolic compounds catechin, epicatichin, taxifolin, apigenin, eriodictyol, luteolin, and naringenin (Sudjaroen *et al.*, 2005).

Hydroxyprolinuria, a candidate biomarker for increased collagen degradation, is a common finding in fluorotic animals (Khandare *et al.*, 2005; Gupta *et al.*, 2012). Enhanced hydroxyproline in urine is due to increased collagen breakdown as a result of disturbances in the formation of collagen cross-links. In the present study, co-administration of *T. indica* fruit pulp extract during oral exposure to fluoride reduced the hydroxyproline concentration in urine. The decreased hydroxyproline concentration in urine of the *T. indica* fruit pulp extract treated group, as compared to fluoride-exposed untreated rats, indicated lower collagen breakdown.

The hydroxyproline concentration of the tibia bone of rats that received *T. indica* fruit pulp extract along with fluoride was comparable with that of healthy control rats, suggesting its ameliorative effects on fluoride induced collagen degradation. Decreased hydroxyproline in the tibia bone could be due to decrease in the biosynthesis of collagen or increase in the degradation of collagen (Shanthakumari & Subramanian, 2007; Gupta *et al.*, 2013a; Gupta *et al.*, 2015a). The plasma and tissue level of ascorbic acid were reported to be decreased in experimental fluorosis (Chinoy *et al.*, 2005; Jhala *et al.*, 2008). Ascorbic acid is a cofactor for the enzyme prolyl hydroxylase, required for the hydroxylation of proline forming hydroxyproline. The protective effect of tamarind extract on tissue collagen by maintaining the activity of prolyl hydroxylase and the decrease in the rate of collagen degradation might be due to the presence of ascorbic acid in the extract (Coronel, 1999).

Supplementation of protein, calcium and ascorbic acid in diet has been suggested to increase the expression level of Col1a1 gene in fluoride-exposed rabbits (Yan *et al.*, 2007). Proanthocyanidines stabilize and increase the cross-linkage of type 1 collagen fibrils by hydroxylation of proline, which is an essential step of collagen biosynthesis (Manimaran *et al.*, 2011). In fluoride-exposed rats, the present study revealed down-regulation of the expression level of the Col1a1 gene by 57% on day 90. Co-administration of the tamarind fruit pulp extract prevented the down-regulation of the expression of type 1 collagen gene as compared to fluoride-exposed rats, indicating the beneficial effect of *T. indica* fruit pulp extract against fluoride toxicity. The ameliorative potential for up-regulation of expression of type 1 collagen gene might be due to the presence of high concentration of protein, calcium, ascorbic acid and proanthocyanidines in the extract of the tamarind fruit pulp (Coronel, 1999).

The extract of the tamarind fruit pulp significantly reduced the bone fluoride concentration. Co-administration of an extract of tamarind pulp with fluoride resulted in an overall 17.66% reduction in tibia bone fluoride concentration at the end of the experiment. Copper has been reported to prevent fluoride accumulation in bone (Khandare *et al.*, 2005). The highest concentration of copper was found in the tamarind fruit pulp extract which might have contributed towards reduced bone fluoride deposition in the rats receiving the tamarind extract. Iron and zinc form insoluble complexes with fluorine at gut level and interrupt its absorption. The presence of these trace elements in the tamarind extracts might be additionally responsible for reduction of the body fluoride burden.

There was significant reduction in the concentration of calcium and increase in the concentration of phosphorus in plasma of fluoride exposed rats. The hypocalcemia observed in the present study might be due to decreased calcium absorption from the gut, increased calcium accumulation in tissues and/or parathyroid gland stimulation (Verma and Guna Sherlin, 2002). Increase in plasma phosphorus has been noticed in fluoride intoxicated animals (Ranjan *et al.*, 2009; Gupta *et al.*, 2013b; Gupta *et al.*, 2015a). The change in serum ALP activity following fluoride administration reflected toxicity of fluorine to osteoblasts (bone forming cells) as well as resorbing osteocytes (Krook & Minor, 1998). It is suggested that the failure in repair response involving an initial increase in formation and resorption of bone is responsible for the increase in serum ALP activity. As the repair process fails, there is toxic death of resorbing osteocytes and decrease in bone resorption, which leads to high ALP activity in serum (Krook & Minor, 1998). Increase in ALP activity is almost a consistent finding in natural as well as experimental fluorosis in animals irrespective of the species involved (Patra *et al.*, 2000; Shanthakumari *et al.*, 2004; Gupta *et al.*, 2015a). Fluoride induced hypocalcemia, hyperphosphatemia and increased serum alkaline phosphatase activity were reversed variably by co-administration of tamarind fruit pulp extract. This beneficial role could be either due to their body fluoride reducing ability or combined effects exerted by various phyto-constituents present in the extract.

The present experiment provides preliminary information on the potential of tamarind fruit pulp extract in alleviating fluoride induced collagen degradation in rats. Therefore, incorporation of tamarind fruit pulp as feed supplement for animals living in fluoride endemic areas is advocated to provide protection from the toxic effects of fluoride, supplying some additional nutrients for improving health.

Acknowledgements

The authors are thankful to Mr. Brijesh Tyagi for his technical assistance.

Conflict of interest statement. *The authors declare that there is no conflict of interest.*

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