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Moringa oleifera AMELIORATES THE EXPRESSION OF COLLAGEN-1 IN TIBIA BONE OF FLUORIDE-INTOXICATED RATS

Amit Raj Gupta[2*], Sahadeb Dey, Mohini Saini[2] and Devendra Swarup

Environmental Medicine Laboratory, Division of Medicine, Indian Veterinary Research Institute, Izatnagar- 243 122, Uttar Pradesh (India)
[1]College of Veterinary Science and Animal Husbandry, Orissa University of Agriculture and Technology, Bhubaneswar – 751 003, Odisha (India)
[2]Centre for Wildlife, Indian Veterinary Research Institute, Izatnagar - 243 122, Uttar Pradesh (India)
*e-mail: *dramitrajgupta@gmail.com*

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ABSTRACT

The present study was aimed to assess the protective effect of *Moringa oleifera* fruits extract against sodium fluoride induced down-regulation of collagen-1 gene in tibia bone of rats. Twenty four albino rats were randomly assigned to four groups. The 1st group served as control and received only tap water. The 2nd group received sodium fluoride (200 ppm) through drinking water. The 3rd group received extract of *M. oleifera* alone and 4th group received *M. oleifera* extract alongwith fluorinated drinking water, daily by gavage for 8 weeks. Significant decrease in the expression of collagen-1 gene in tibia bone was noted in fluoride treated rats. Supplementation of *M. oleifera* fruits extract during exposure to fluoride up-regulated the expression of collagen-1 gene in tibia bone as compared to untreated fluoride exposed rats. It is concluded that daily oral administration of *M. oleifera* fruit extract has positive influence on the expression of collagen-1 gene in bones of fluoride intoxicated rats.

Keywords: Collagen-1, gene expression, fluoride, *Moringa oleifera*, rats

INTRODUCTION

Fluorosis is an endemic public health problem in 23 nations around the globe and causes dental, skeletal and non-skeletal fluorosis (WHO, 2002). The skeletal fluorosis affects bones and joints so is not easily recognisable until the disease reaches advanced stage. Fluoride disrupts collagen synthesis resulting in the formation of imperfect collagen and/or non-collagenous protein in cells (Sharma, 1982). Collagen is a major structural protein in extracellular matrix of musculoskeletal tissues and constitutes about 90% of total protein in tendons and bones as well as in substantial part of connective tissues in skeletal muscles (Smith and Rennie, 2007). Type 1 collagen is the principal extracellular matrix protein in bone and teeth (George *et al*., 1999). Many studies have shown that fluoride negatively affects collagen metabolism and leads to the breakdown of collagen in bone, tendon, muscle, skin, cartilage, lung, kidney and trachea of rabbits (Susheela and Mukerjee, 1981) and in skeletal muscles of rats (Gupta *et al*., 2013). Earlier studies have shown significant negative effects of fluoride on type 1 collagen (Col1a1) in the ribs of rabbits (Yan *et al*., 2007), teeth of sheep and guinea pigs (Han *et al*., 2010, Wang *et al*., 2010) and muscles of rats (Gupta *et al*., 2013).

Herbal medicines are traditionally used worldwide for the prevention and treatment of various diseases. In India, medicinal plants are widely used in ayurvedic medicines for the treatment of various health problems (Jain, 2000). Enhanced urinary fluoride excretion and reduction of fluoride associated oxidative stress have been reported in rabbits (Ranjan *et al*., 2009) and rats (Dey *et al.*, 2011). *Moringa oleifera* Lam. (family: *Moringiaceae*), commonly known as horseradish /drumstick tree, is most common species cultivated in tropical areas (Vlahov *et al*., 2002, Manzoor *et al*., 2007). The plants is a rich source of β-carotene, protein, vitamin C, calcium, potassium and natural anti-oxidants (Kawo *et al*., 2009); and enhance the shelf-life of fat-containing foods due to the presence of various types of antioxidant compounds like ascorbic acid, flavonoids, phenolics and carotenoids (Dillard and German, 2000, Siddhuraju and Becker, 2003). Earlier studies in rats (Stanley *et al.*, 2002) and rabbits (Ranjan *et al*., 2009) have suggested its beneficial effect in fluoride toxicity by way of increased urinary fluoride excretion and decreased fluoride retention in bone. However, the efficacy of *M. oleifera* on fluoride induced collagen degradation has never been assessed. Therefore, the present study was aimed to assess the ameliorative effect of hydro-methanolic extract of *M. oleifera* fruits on the expression of collagen-1 gene in tibia bone of fluoride intoxicated rats.

MATERIALS AND METHODS

The present study was conducted at the Environmental Medicine Laboratory, Division of Medicine, IVRI, Izatnagar (India) in the year 2010. Unripe pods of *Moringa oleifera* were procured from local market in Bareilly, UP (India). The identity of plant was authenticated by Botanical Survey of India, Central National Herbarium, Howrah, India vide Voucher specimen No. CNH/I-I(174)/ 2007/ Tech.II/104).

Preparation of plant extract

The plant material was washed with distilled water to remove any dirt, air-dried and powdered. The powdered material (100 g) was mixed with 1.5 L solvent (methanol: water, 1:1) and kept overnight at 25ºC. Then, it was stirred in a magnetic stirrer for 1 h and filtered through Whatman filter paper No. 1. The extract was subjected to *in vacuo* solvent evaporation in a rotary evaporator under reduced pressure. A pasty material was obtained (25 g) and stored at 4ºC until use (Ranjan *et al*., 2009).

Animals and experimental design

Female 8 week old Wistar albino rats (n = 24), body weight 120-130 g, were procured from Laboratory Animal Resource Section, IVRI, Izatnagar (India). The experiment was performed with approval from Institute Animal Ethics Committee (Approval No.: 1-53/2004-JD-Research dated 09.04.2010). Rats were housed in polypropylene cages under 12 h dark/12 h light cycle with temperature in animal house ranging from 18 to 25°C and humidity between 55 and 60%. Throughout the study, the rats were provided *ad libitum* access to tap water containing 0.23 ppm fluoride and laboratory ration consisting of 12% wheat bran, 87% maize, 1% mineral salt, and 4.2 ppm fluoride.

After 15 days of acclimatization period, the animals were randomly assigned in 4 groups of 6 rats each. Group I served as control and received only tap water. Group II received 200 ppm sodium fluoride (NaF, 99% pure, Qualigens Chemicals, Mumbai, India) through drinking water. Group III received hydro-methanolic extract of *M. oleifera* fruits (200 mg kg^{-1} body weight) and group IV received *M. oleifera* fruits extract + fluorinated (200 ppm) drinking water, daily by gavage for 8 weeks. The selected dose of sodium fluoride to induce toxicity was based on published literature (Yan *et al*., 2007) and earlier studies conducted in our laboratory (Ranjan *et al*., 2009, Dey *et al.*, 2011). The selected dose of *M. oleifera* was based on previous studies (Ranjan *et al*., 2009).

Sample collection

After eight weeks, the animals were deprived of food overnight and sacrificed by decapitation. Tibia bones were harvested and any attached tissue quickly removed from the bone using a scalpel. The bone was snap frozen in liquid nitrogen and stored at -80°C for total RNA extraction.

Reverse transcriptase (RT) and real-time PCR for collagen

Tibia bone, stored at -80°C, was transferred to liquid nitrogen and crushed to powder. The powdered material was transferred to 1.0 mL Trizol reagent (Life Technologies, USA) and homogenized to isolate the total RNA as per the protocol of Yan *et al*. (2007). RT was performed using the first strand cDNA synthesis kit (Fermentas, Life Sciences) in a volume of 20 µL containing 5 µg total RNA, 0.5 µg oligo dT primer, 20 units of RiboLock^{TM} ribonuclease inhibitor, 10 mM dNTP mix, 40 units of M-MuLV reverse transcriptase in 5X reverse transcriptase buffer. The reaction mixture was incubated at 37°C for 1 h. The cDNA synthesis was confirmed by amplifying the Gapdh amplicon in PCR. Quantitative real-time PCR assay was performed with Brilliant® SYBR® Green Master Mix (Stratagene, USA) and Mx3000P spectrofluorimetric thermal cycler operated by MxPro^{TM} QPCR software. Aliquots of RT reactions were subjected to PCR in 20 µL reactions with SYBR® Green QPCR Master Mix using primers for Col1a1 and Gapdh (Table 1). No template control was put for either gene quantification for checking the contamination in reaction components other than cDNA. Non-reverse transcribed RNA (10 ng) 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 95°C for 10 min., denaturation at 94°C for 30 sec., annealing at 55°C for 30 sec., extension at 72°C for 45 sec for 40 cycles. Fluorescence was recorded at the end point of each cycle and dissociation (melting) curve consisting of 95°C for 30 sec, followed by 55°C for 30 sec and gradual increment from 55 to 95°C at 2°C per min. and lastly 95°C for 30 sec. Relative quantification of quantitative real time PCR product was performed using comparative $\Delta\Delta C_T$ method (Pfaffl, 2001). The results of Real time PCR were depicted as fold change of Col1a1 mRNA level in tibia bone of experimental rats with normal rats.

Statistical analysis

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 with $p<0.05$ considered statistically significant.

RESULTS AND DISCUSSION

The expression level of Col1a1 gene calculated by $\Delta\Delta C_T$ method in rats of different groups is shown in Fig. 1. The expression level of collagen-1 gene in tibia bone of control group and the group that received *M. oleifera* fruits extract alone were similar. The result showed decreased expression of type 1 collagen gene in fluoride exposed groups that increased significantly ($p<0.05$) with concomitant use of *M. oleifera* fruits extract.

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' (forward) 5'-AAGTCCATGTGAAATTGTCTCCCA-3' (reverse)	4454-4599	136	NM_053304
Gapdh	5'-ACATCATCCCTGCATCCACT-3' (forward) 5'-TTTCTCCAGGCGGCATGTCA-3' (reverse)	684-823	140	NM_017008.3

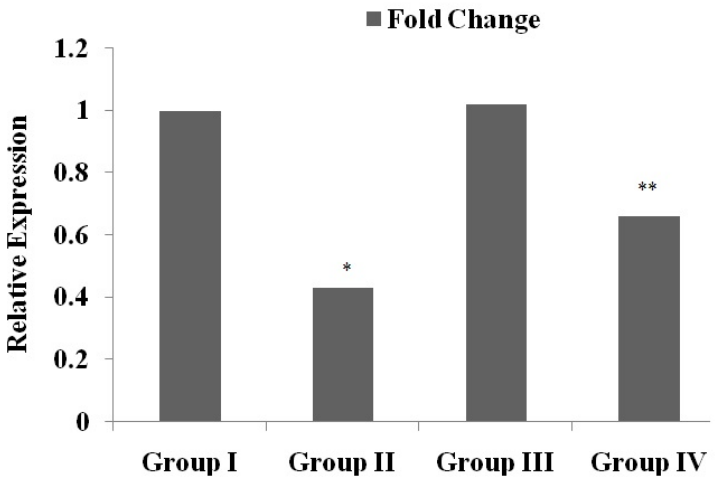

Fig. 1: Quantitative real time PCR analysis of the effect of sodium fluoride treatment on Col1a1 mRNA expression in tibia bone of rats. Relative mRNA abundance was calculated using $\Delta\Delta C_T$ method. Expression level of Col1a1 mRNA was normalized relative to that of Gapdh mRNA. Group I = control; Group II = fluoride exposed rats; Group III = *M. oleifera* fruit extract and Group IV = fluoride + *M. oleifera* fruit extract; $^{*}P_{0.05}$ compared with healthy control; $^{**}P_{0.05}$ compared with fluoride exposed rats.

Co-administration of hydro-methanolic extract of *M. oleifera* fruits along with sodium fluoride for 8 weeks resulted in variable degrees of protection from fluoride induced down-regulation of collagen-1 gene in tibia bone of rats. Supplementation of protein and Ca in diet has been suggested to increase the expression level of Col1a1 gene in fluoride exposed rabbits (Yan *et al.*, 2007). The present study revealed down regulation of expression level of Col1a1 gene by 57% in fluoride exposed rats after 8 weeks. Co-administration of *M. oleifera* fruits extract along with fluoride prevented down-regulation of expression of Col1a1 gene as compared to fluoride exposed rats, indicating the beneficial effect of *M. oleifera* fruits extract against fluoride toxicity. The plasma and tissue level of ascorbic acid have been reported to decline in experimental fluorosis (Jhala *et al.*, 2008). Ascorbic acid is a cofactor for the enzyme prolyl hydroxylase, required for hydroxylation of proline forming hydroxyproline. Beneficial effect of *M. oleifera* fruits extract on tissue collagen might be due to the presence of high concentration of protein, calcium and ascorbic acid in the extract (Kawo *et al*., 2009), thus maintaining the activity of prolyl hydroxylase enzyme and decrease the rate of collagen degradation in tibia bone.

Moringa seeds contain a cationic polyelectrolyte that has proved efficient in water treatment as a substitute to aluminium sulphate or other flocculent (Ndabigengesere and Narasiah, 1998). The beneficial effects of fruits with seed extracts can be attributed to the prevention of fluoride absorption due to its flocculent action. Since protein, calcium and ascorbic acid have been reported to ameliorate the fluoride toxicity (Wang *et al*. 2008), the ameliorative effect of *Moringa oleifera* fruit extracts in terms of up-regulation of collagen-1 gene expression could be attributed to its high content of protein, calcium and ascorbic acid as reported earlier. On the basis of above observation, it is clear that *M. oleifera* fruit extract has a positive effect on collagen content and its characteristics in fluoride exposed rats. Further research on the fractionation of active compound and synergistic effects in different plant extracts needs to be explored to determine the interactions among phytochemicals presents in the extracts.

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