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Beetroot Juice Supplementation Alleviates Adverse Effects of Ingested Fluoride

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Abstract

The protective effect of beetroot juice supplementation against oxidative stress induced by oral administration of sodium fluoride, (NaF-12 ppm in drinking water), was evaluated. Male Sprague Dawley rats, weighing 200-250g, were divided into three groups i.e. sham control, fluoride exposed (12 ppm in drinking water), fluoride exposed (12 ppm in drinking water) and beetroot juice supplemented (0.5 ml / rat/ day) rats. Ingestion of fluoride water increases free radical production which inhibits the enzyme activities and resulted in altered serum biochemistry. Levels of hydroperoxides in liver were found to be decreased in beetroot juice supplemented group as measured by levels of thiobarbituric acid reactive substance (TBARS). Activities of superoxide dismutase (SOD), glutathione reductase (GR), glutathione S-transferase (GST), increased in beetroot juice supplemented group in comparison to sham control and fluoride exposed group. Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were found to be increased in liver by beetroot juice supplementation. Reduced glutathione (GSH) to oxidized glutathione (GSSG) ratio was found to be increased in beetroot juice supplemented group in comparison with sham control and fluoride exposed group. Comet assay results show that beetroot juice supplementation also protects from DNA damage caused by fluoride exposure. The altered enzymatic activities and biochemical parameters were restored almost to control level. In conclusion, beetroot juice supplementation played a beneficial role in protection against fluoride induced DNA damage and oxidative stress.

Introduction

Fluoride (F) has a strong tendency to acquire a negative charge as it is highly electronegative and readily forms fluoride ions [1]. Fluoride toxicity is generally associated with the deliberate fluoridation of public drinking water supply [2]. At 2.6 ppm F, most children and adults showed evidence of dental fluorosis [3]. About 40% of ingested fluoride is absorbed from the stomach as hydrogen fluoride. Intracellular intake of HF is more pronounced as the penetration of small neutral molecule of HF is faster than the dissociated fluoride ion [4]. This ingested fluoride then combines with cations of calcium, magnesium etc. to form insoluble complexes that can cause hypocalcemia and inhibit magnesium and manganese-dependent enzymes [1]. Fluoride combines with metallic ions such as aluminium (Al) or beryllium (Be) to form inorganic complexes which can have adverse biological effects and may even prove to be toxic [4].

Exposure to fluoride increase generation of anion superoxide ($O2^-$) [5,6], which further mediates fluoride toxicity by generation of other species such as hydrogen peroxide, peroxinitrite, hydroxyl radicals [7-9]. Oxidative stress has been observed in vitro as well as in vivo in soft tissues such as liver, kidney, brain, lungs, and reproductive system after fluoride exposure [10,11]. Excessive production of reactive oxygen species (ROS) leads to macromolecule oxidation [12], resulting in membrane damage via induction of lipid peroxidation, mitochondrial membrane depolarization, and apoptosis [13].

Several studies have been conducted on the prevention of the potential effects of fluoride by means of administration of vitamins, various minerals and other compounds [3,14-16]. Supplementations with herbal antioxidants like black berry juice [17], spirulina, tamarind fruit pulp extract [18], have been reported to be beneficial in preventing oxidative damage in experimental animals exposed to high fluoride level[13,19]. Beetroot has betalain pigments which belong to group of cation antioxidants. Betalains are coloured, water-soluble nitrogen compounds found in cell sap [20]. Betalains have a wide range of desirable biological activities, including antioxidant, anti-inflammatory, hepatoprotective, anti-cancer properties [21, 22, 23].

However there is a paucity of literature on alleviation of adverse effects generated by fluoride exposure through beetroot juice supplementation. The present study was undertaken with two main objectives viz., first to analyze level of oxidative stress for short duration exposure with low doses (12ppm for 7 days) of fluoride intake. Secondly, to assess the effect of beetroot juice on the adverse effects generated by deliberate administration of fluoride to rats, by evaluating changes in biochemical parameters pertaining to liver tissue, leg muscle, plasma and DNA damage in spleen and bone marrow cells.

Materials and methods

Experimental Animal

The healthy, adult albino rats (***Rattus norvegicus***) of Sprague dawley strain weighing between 200-250 gm were used for experiments. Each rat was housed in separate cage at animal facility of the institute. The rats were provided

with ad libitum feed and water. The temperature of the animal house was maintained at 22-24°C. The animals were exposed to 12h light/12h dark cycle.

Beetroot juice

Beetroot juice used in this study was a product of Defence Food Research Laboratory (DFRL), Mysore.

Experimental Design

The study was carried out on twelve rats divided into three groups i.e. group 1- Sham Control, group 2- fluoride exposed and group 3- fluoride exposed along with beetroot juice supplemented. The first group that served as sham control group, was administered normal drinking water (i.e. without sodium fluoride (NaF)) and 10% sucrose supplementation (0.5ml each rat) for 14 days as the sugar content of juice was found to be 10%. The second group named, fluoride exposed group was supplemented with normal drinking water (i.e. without added NaF) and 10% sucrose (0.5ml each rat) for initial seven days prior to fluoride exposure; followed by administration with fluoridated water (12ppm NaF) and 10% sucrose (0.5ml each rat) over the next seven days. The third group was given normal drinking water and beetroot juice (0.5ml each rat) for first seven days and fluoridated water (12ppm NaF) along with beetroot juice (0.5ml each rat) for next seven days. Body weight and food intake were monitored during the study period of 14 days. Fluoride exposure was made through drinking water available ad libitum while sucrose solution and beetroot juice were fed orally using feeding canula.

The research was approved by animal ethics committee of the institute as a part of project S&T-09/DIP-251.

Tissue collection and preparation of homogenates

On 15th day of the aforesaid treatment, animal were fasted overnight and sacrificed under ether anesthesia. Blood samples were collected into heparinized tubes by cardiac puncture. The blood samples were centrifuged at 3000 rpm for 10 minutes for separation of plasma and cells. After cntrifugation, packed RBCs were collected from bottom of the tube and washed with 150mM KCl. For enzymatic analysis 10% (v/v) heamolysate was prepared using chilled water.

Liver and muscle tissue of all the animals was taken and washed with normal saline, and subsequently blood, fatty parts and connective tissue were removed. Afterwards, tissue homogenates (10% w/v) were prepared using 150mM potassium chloride. It was followed by centrifugation at 3000rpm for 10 minutes. The supernatant were stored in aliquots at -80°C for further analysis.

Determination of protein and oxidative stress markers

The method described by Lowry et al. [24], and modified by Miller [25] was used to determine the protein level in plasma, liver tissue and muscle homogenates. The results were expressed as mg-protein/ml blood in plasma and mg-protein/g tissue. Lipid peroxidation was measured using TBARS [26]. The specific activities of enzymes viz. glutathione S transferase (GST) [6,27], glutathione peroxidase (GPx) [28], glutathione reductase (GR) [29], superoxide dismutase (SOD) [30], alanine aminotransferase (ALT) [31], aspartate aminotransferase (AST) [31] were also measured. The reduced glutathione (GSH) and oxidised glutathione (GSSG) were measured using method described by Hissin and Hilf, 1976 [19].

Comet Assay

The single cell gel electrophoresis or comet assay was used to detect DNA damage caused by intake of fluoridated water using the method of Östling & Johansson (1984) with modifications by Singh et al. [32]. Cells embedded in agarose on a microscope slide were lysed with triton X-100 under alkaline conditions. The structures resembling comets formed due to electrophoresis at high pH were observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks. This was followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage [32, 33].

Interpretation of the results was done using origin 6.0 professional software. Comet assay result was analysed using comet IV software.

Results and discussion

Body weight and food intake:

The data revealed that body weight gain in fluoride (12ppm) water treated rats for seven days is less in comparison with sham control rats. The increase in body weight over 14 days of treatment was observed in all the three groups i.e. 52 g, 43 g, 49 g respectively as shown in table 1. Fluoride ions exert deleterious effects on some nutrient utilization on experimental animals resulting in poor growth. However, beetroot juice supplementation helps in restoring weight and thereby weight gain in beetroot juice supplemented group is more as compared to fluoride exposed rats. The food intake

of fluoride exposed was found to be decreased after 14 days, whereas in sham control and beeroot juice supplemented group food intake remains almost same.

Table 1: Body weight and food intake of sham control, fluoride (12ppm) toxicity and Fluoride toxicity + Beetroot juice supplemented rats.

Groups	Mean Body Weight (g)		Mean Food Intake (g)	
	Initial	After 14 days	Initial	After 14 days
Sham Control Rats (n=4)	196±16	248±24	15.7±2.2	16±2.1
Fluoride exposed Rats (n=4)	207±11	250±26	17.2±2.8	13±1.6
Fluoride exposed + Beet Root Juice supplemented Rats (n=4)	187± 23	236±19	14.7±1.7	14±1.0

Oxidative stress markers - Enzyme activity and biochemistry:

As reported in previous studies, there is decrease in the activity of antioxidant enzymes like superoxide dismutase, glutathione reductase while some other enzymes like glutathione peroxidase, glutathione S transferase show increased activities after long term exposure with high dose (50-200ppm) of sodium fluoride [5,32]. However in the present study, activities of antioxidant enzymes were found to be increased in fluoride exposed group as compared to sham control group. This increase in the enzyme activity may be a protective response due to short term fluoride exposure (low dose-12ppm).

The activity of superoxide dismutase increased in fluoride exposed group (6.8 ± 0.6 U/ml) as compared to sham control group (6.0 ± 2.2 U/ml). Same was in case of Beetroot juice supplemented and fluoride exposed group (6.7 ± 0.9 U/ml) as shown in Figure 1(a).

Beetroot juice supplementation significantly increases enzymatic activity of glutathione reductase in comparison with sham control and fluoride exposed group as shown in Figure 1(b).

Glutathione peroxidase activity of fluoride exposed group was higher in comparison to sham control group both in case of liver and muscle. Beetroot supplementation showed an increase in glutathione peroxidase activity of liver and a decrease in muscles. The difference in glutathione peroxidase activity of liver and muscle could be attributed to the higher metabolic activity of liver as shown in Figure 1(c).

Glutathione S transferase activity was found to be higher in fluoride exposed group as compared to sham control group in both liver and muscle tissue homogenates. In comparison with sham control group, supplementation of beetroot juice resulted in increased activity of glutathione S transferase in liver as shown in Figure 1(d). However, reverse pattern is observed in muscle.

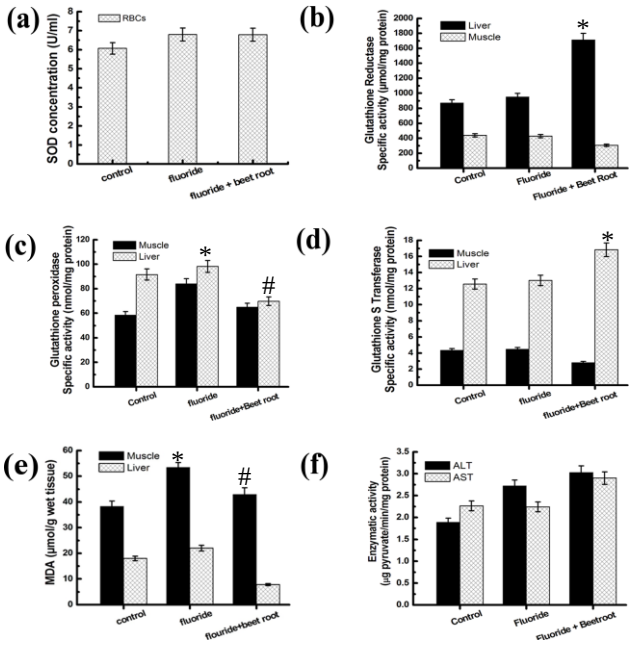

Figure 1. Protective effects of beetroot juice supplementation on fluoride exposed rats. (a) Activity of Superoxide Dismutase (SOD) in RBCs (U/ml). (b) Activity of Glutathione Reductase in liver and muscle tissue homogenate (µmol/mg protein, *p<0.05 in comparison with sham control). (c) Activity of Glutathione peroxidase in liver and muscle samples (nmol/mg protein; *p<0.05 in comparison with sham control, #p<0.01 in comparison with fluoride exposed). (d) Activity of Glutathione S Transferase in liver and muscle (nmol/mg protein; *p<0.05 in comparison with sham control). (e) Lipid peroxidation measured by TBARS method (µmol/g wet tissue; *P<0.05 in comparison with sham control, #p<0.01 in comparison with fluoride exposed). (f) Enzymatic activity of Alanine aminotransferase (ALT) aspartate aminotransferase (AST) in liver.

Studies have reported that excessive production of ROS due to fluoride intake leads to macromolecule oxidation, resulting in membrane damage due to lipid peroxidation [13]. Estimation of levels of malondialdehyde (MDA) was used to estimate lipid peroxidation. In liver and muscle tissue homogenates, highest lipid peroxidation was observed in fluoride exposed group as shown in Figure 1(e) and lowest in the group that was supplemented with beetroot juice. Therefore, it can be said that beetroot supplementation shows ameliorative effect against fluoride toxicity in comparison with both sham control and fluoride toxicity groups.

In liver, fluoride intake resulted in increased enzymatic activity of both ALT and AST as shown in Figure 1(f). But beetroot supplemented group shows further increase in activity of these enzymes in comparison with sham control and fluoride exposed group. Hence, it infers that beetroot juice supplementation shows hepatoprotective activity by improving ALT and AST in liver.

It has been reported that fluoride can alter glutathione levels often resulting in excessive production of ROS at mitochondrial level, leading to damage of cellular components [34, 35, 36]. Glutathione levels of blood, liver and muscle have been shown in Table 2. A decrease in GSH:GSSG ratio was observed in fluoride toxicity group which shows increase in oxidative stress due to intake of fluoridated water. Moreover, beetroot supplementation was found to be effective in ameliorating the effect of fluoride exposure in blood samples as the GSH:GSSG ratio increased in the beetroot supplemented group.

Table 2: GSH:GSSG Ratio in blood (µmol/ml), liver (µmol/g tissue wt.) and muscle (µmol/g tissue wt.) tissue homogenates.

	Blood (µmol/ml)	Liver (µmol/g tissue wt.)	Muscle (µmol/g tissue wt.)
Sham Control	3.98±0.87	28.4±8.27	5.0±1.24
Fluoride exposed	3.91±0.82	27.3±7.63	4.6±1.53
Fluoride exposed + Beetroot supplemented	4.24±1.09	29.9±8.52	4.9±1.64

DNA Damage:

The comet assay, which is also referred to as the single cell gel electrophoresis assay (SCG or SCGE assay), is a rapid and quantitative technique by which visual evidence of DNA damage in eukaryotic cells can be measured. It is based on quantification of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis, analysed under fluorescent microscope. The study revealed that fluoride toxicity induced DNA damage as comet tail was observed in spleen cells and bone marrow cells of fluoride treated rats. Further, beetroot supplementation exhibited an ameliorative effect against fluoride intake as no comet tail was observed in beetroot supplemented group. Figure 2(a) shows results of comet assay with bone marrow cells as sample. The spleen cell DNA damage has been shown in Figure 2(b).

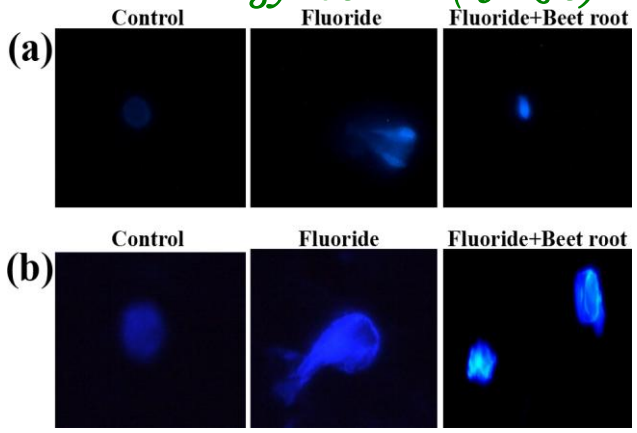

Figure 2. Evaluation of DNA damage by comet assay (a) Bone marrow cells and (b) Spleen cells.

It was observed from the previous studies [37] that sacrificial antioxidants from diet reduce the severity and incidence of fluoride induced toxicity. Food rich in protein, vitamins, essential amino acids and minerals exhibited protection from fluoride induced oxidative stress to various organs in rats [38].

Conclusion

Our results revealed that even low doses of fluoride (12ppm) ingestion for short period of time significantly decreased animal growth which may be due to decreased feed consumption. Fluoride had significant adverse effects on plasma indices in rats. However, the administration of beetroot juice to fluoride treated rats attenuates the fluoride induced dysfunctions. The administration of fluoride to rats at the dose of 12ppm for a period of 7 days led to changes in the levels of MDA and activities of SOD, GR, GST, GPx, AST and ALT. Beetroot juice supplementation was effective in ameliorating the adverse changes in MDA levels, activities of SOD, GPx, GR, GST caused by fluoride toxicity. Fluoride exposure induced DNA damage as comet tails were seen in fluoride exposed group. Beetroot juice supplementation ameliorated the effects of fluoride toxicity on DNA damage as the group that was administered with beetroot juice in association with fluoridated water did not exhibit comet tail. Hence, oral administration of beetroot juice could alleviate the adverse effect of fluoridated water. There is need for further study on human participants living in endemic areas of fluorosis to assess effect of natural antioxidants from fruits and vegetables.

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