Protective effect of dietary flaxseed oil on arsenic-induced nephrotoxicity and oxidative damage in rat kidney

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A B S T R A C T

Arsenic, a naturally occurring metalloid, is capable of causing acute renal failure as well as chronic renal insufficiency. Arsenic is known to exert its toxicity through oxidative stress by generating reactive oxygen species (ROS). Flaxseed, richest plant based dietary source of ω-3 polyunsaturated fatty acids (PUFAs) and lignans have shown numerous health benefits. Present study investigates the protective effect of flaxseed oil (FXO) on sodium arsenate (NaAs) induced renal damage. Rats prefed with experimental diets (Normal/FXO diet) for 14 days, were administered NaAs (20 mg/kg body weight i.p.) once daily for 4 days while still on the experimental diets. NaAs nephrotoxicity was characterized by increased serum creatinine and blood urea nitrogen. Administration of NaAs led to a significant decline in the specific activities of brush border membrane (BBM) enzymes both in kidney tissue homogenates and in the isolated membrane vesicles. Lipid peroxidation and total sulfhydryl groups were altered upon NaAs treatment, indicating the generation of oxidative stress. NaAs also decreased the activities of metabolic enzymes and antioxidative defense system. Histopathological studies supported the biochemical findings showing extensive damage to the kidney by NaAs. In contrast, dietary supplementation of FXO prior to and along-with NaAs treatment significantly attenuated the NaAs-induced changes.

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1. Introduction

Human exposure to metals/metalloids such as uranium, lead, arsenic (As) and cadmium in both occupational and environmental settings is a common occurrence. Arsenic is a metalloid found in water, soil and air from natural and anthropogenic sources and exists in inorganic as well as organic forms (Flora et al., 2009). The major inorganic forms of arsenic include trivalent arsenite (As(III)) and pentavalent arsenate (As(V)). Arsenic typically arsenate enters the body mainly via consumption of contaminated drinking water (Shi et al., 2004). Prolonged ingestion of water contaminated with arsenic result in the manifestations of toxicity in practically all systems of the body (Mandal and Suzuki, 2002).

Kidney is a known target organ for arsenic and is critical for both arsenic biotransformation and elimination. Epidemiologic investigations and animal experiments have demonstrated that acute and chronic exposure to arsenic can cause injury to the kidney and increase the risk of renal cancer (Waalakes et al., 2004). The epithelial cells of proximal convoluted tubules are found to be more sensitive to arsenic induced toxicity due to their high reabsorptive activity and anatomical positions as the first renal tubular epithelial cells to be exposed to filtered toxicants (Peraza et al., 2006). Combined ultrastructural/biochemical studies (Brown et al., 1976) conducted in kidneys of rats exposed to arsenate have shown in situ swelling of mitochondria associated with decreased respiratory functions. Moreover, arsenic has been reported to increase the number of lysosomes (Brown et al., 1976). Increasing evidence indicate that multifactorial mechanisms might be involved in metal induced toxicity and that one of the well known mechanism involves metal induced reactive oxygen species (ROS) generation (Abdel Moneim et al., 2011; Kokilavani et al., 2005). Arsenic is one of the most extensively studied metals that induce ROS generation and results in oxidative stress (Roy et al., 2009). Interference of toxic metals with glutathione (GSH) metabolism is found to be an essential part of the toxic response of many metals (Scott et al., 1993). Depletion of cellular sulfhydryl reserves seems to be an important indirect mechanism for arsenic-induced oxidative stress (Stohs and Bagchi, 1993). Consequently in studies aimed at abrogation of arsenic toxicity, the generation of oxidative stress has been targeted and the beneficial effects of various ROS scavengers (Flora, 1999; Ramanathan et al., 2005) have been explored. Certain complexing and metal chelating agents were also used to reduce arsenic induced toxicity (Gupta et al., 2004).
However, most of these agents have been reported to exhibit toxic manifestations (Shi et al., 2004). This has led to an increased interest in utilizing the therapeutic potential of naturally occurring dietary nutrients having free radical scavenging and/or antioxidant properties to counteract free radical mediated arsenic toxicity (Manna et al., 2008). One such source of dietary nutrient is flaxseed. Flaxseed meal and flaxseed oil (FXO) have been used as/ in food for centuries in Asia, Europe and Africa. Recently, flaxseed (Linum usitatissimum) has been identified as the richest plant based dietary source of omega-3 (α-3/ω-3) polyunsaturated fatty acids (PUFAs), specifically α-linolenic acid (ALA). Nutritional recommendations have emphasized the need to consume α-3 PUFAs owing to their several beneficial effects on human health. Further, a number of animal studies involving dietary supplementation of flaxseed/FXO have reported inhibition of arhythrogenesis during ischemia–reperfusion (Ander et al., 2004), inhibition of atherosclerosis (Prasad, 2005) and protection against vascular dysfunction during hypercholesterolemic conditions (Dupasquier et al., 2006). The activity of constituent ALA itself or of its longer-chain PUFA derivatives viz. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) might be playing an important role in these healing effects (Sinclair et al., 2000; Burdge and Calder, 2005). Flaxseed is also a good source of dietary fiber and phytosterogenic lignans that are believed to have antioxidant properties (Newairy and Abdou, 2009; Zanwar et al., 2010). The oxygen radical scavenging properties of flaxseed lignans were shown in vitro by either direct hydroxyl radical scavenging activity or inhibiting lipid peroxidation (Kitts et al., 1999). We have recently reported that FXO mitigates lead induced nephrotoxic effects (Rizwan et al., 2013). However, the efficacy of FXO to protect against arsenic-induced nephrotoxicity has not yet been evaluated.

In view of this, the present work was undertaken to study detailed biochemical events/cellular response/mechanisms of sodium arsenate (NaAs) nephropathy and its possible mitigation by FXO. We hypothesized that FXO would prevent NaAs-induced adverse effects on kidney due to its intrinsic biochemical and antioxidant properties that would result in improved metabolism and antioxidant defense mechanism in the kidney.

2. Materials and methods

2.1. Chemicals and drugs

Flaxseed oil: Omega Nutrition Canada Inc (Vancouver, BC, Canada), sodium arsenate (Merek, Mumbai, India). All other chemicals used were of analytical grade and were purchased either from Sigma Chemical Corp. or SRL (Mumbai, India).

2.2. Diet

A nutritionally adequate laboratory pellet diet was obtained from Aashirwad Industries, Chandigarh (1544, Sector 38-B, Chandigarh, India). Normal diet (ND) was prepared by crushing the pellets finely and adding vitamin E as DL-α-tocopherol (270 mg/kg chow) to the crushed diet. Flaxseed oil (FXO) diet was prepared by adding 15% flaxseed oil by weight to the normal diet. The diet was stored in airtight containers. Vitamin E was added in order to meet the increased metabolic requirement for antioxidants on a diet high in polyunsaturated fatty acids.

2.3. Experimental design

The animal experiments were conducted according to the guidelines of Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. Adult male Wistar rats (10-12 rats/group) weighing between 150 and 200 g were used in the study. Animals were acclimatized to the animal facility for a week on standard rat chow and allowed water ad libitum under controlled conditions of 25 ± 2 °C temperature, 50 ± 15% relative humidity and normal photoperiod (12 h dark and light). Four groups of rats entered the study after acclimatization (Fig. 1). They were fed on either normal diet (control and NaAs groups) or FXO diet (AsFXO and FXO groups). After 14 days, rats in the two groups (NaAs and AsFXO) were injected with sodium arsenate (NaAs) in distilled water intraperitoneally (20 mg/kg bwt/day) every day for 4 days (four sodium arsenate injections in total). The dose of sodium arsenate selected for this study was much less than the reported LD50 value of sodium arsenate for rats (Franke and Moxon, 1936), and was comparable to the dose used in several previous studies to induce the toxic effects (Hood et al., 1988; Hood et al., 1979, 1987). Since our objective was to study the acute effects of arsenic exposure, hence sodium arsenate was injected in 4 doses, over a short period of time. Animals in the control and FXO group received an equivalent amount of distilled water. The rats were sacrificed on the sixth day after the last NaAs injection under light ether anesthesia. Blood and urine samples were collected and kidneys were removed and processed for the preparation of homogenates and brush border membrane vesicles (BBMV) as described below. Each preparation of BBM/homogenate was made by pooling the tissue (cortex/medulla) from 2 to 3 animals in each group. Thus, the number of animals mentioned above contributed to 4–5 different preparations for each group. Analyses of various parameters were performed simultaneously under similar experimental conditions to avoid any day to day variations.

2.4. Histopathology

Animals were sacrificed under light ether anesthesia. Abdomen was opened and kidneys were removed, cut into pieces and kept in Karnovsky’s fixative for one week (Immersion fixation). Small pieces of tissue samples were processed for paraffin embedding. Sections of 5 μm thickness were cut and stained with haematoxylin and eosin. Light microscopic observations were made under trinocular microscope (Olympus BX-40, Japan). Interesting findings were recorded at the initial magnification of 400×.

2.5. Preparation of homogenates

After the completion of the experiment, the kidneys were removed, decapsulated and kept in ice-cold buffered saline (154 mM NaCl, 5 mM Tris–HEPES, pH 7.5). The cortex was carefully separated from medulla as described earlier (Khandmuri et al., 2005). A 15% (w/v) homogenate was prepared in 0.1 M Tris–HCl buffer, pH 7.5, using Potter–Elvehjem homogenizer (Remi Motors, Mumbai, India); by passing five pulses. The homogenate was centrifuged at 3000 × g at 4 °C for 15 min to remove cell debris and the supernatant was saved in aliquots and stored at −20 °C for assaying free-radical scavenging enzymes and for estimation of total-SH and lipid peroxidation (LPO).

2.6. Preparation of brush border membrane

BBMV were prepared from whole cortex using the MgCl₂ precipitation method as described previously (Khandmuri et al., 2004). Briefly, freshly minced cortical slices were homogenized in 50 mM mannitol and 5 mM Tris–HEPES buffer, pH 7.0 (20 ml/g), in a glass Teflon homogenizer with 4 complete strokes. The homogenate was then subjected to high speed Ultra-Turrax homogenizer (Type T-25, Janke & Kunkel GMBH & Co. Kg, Staufen, Germany) for 3 strokes of 15 s each with an interval of 15 s between each stroke. MgCl₂ was added to the homogenate to a final concentration of 10 mM and the mixture stirred for 20 min on ice. The homogenate was centrifuged at 2000 × g for 10 min in a Beckman centrifuge (J2 MI, Beckman instruments Inc Palo Alto, CA, USA) using JA-17 rotor and the supernatant was then recentrifuged at 35,000 × g for 30 min. The pellet was resuspended in 300 mM mannitol and 5 mM Tris–HEPES, pH 7.4, with four passes by a loose fitting Dounce homogenizer (Wheaton, IL, USA) and centrifuged at 35,000 × g for 20 min in a 15 ml conical tube. The outer white fluffy pellet of BBM was resuspended in small volume of buffered 300 mM mannitol. Aliquots of BBM and homogenates were saved and stored at −20 °C for BBM enzyme analysis.

2.7. Serum/urine chemistry

Serum samples were deproteinized with 3% trichloroacetic acid in a ratio of 1:3, left for 10 min and then centrifuged at 2000 × g for 10 min. The protein free supernatant was used to determine inorganic phosphate (Pi) and creatinine. The precipitate was used to quantify total phospholipids. Blood Urea Nitrogen (BUN) was determined directly in serum samples. All these parameters were determined by standard procedures as mentioned in a previous study (Khandmuri et al., 2005). Glucose was estimated by o-toluidine method using kit from Span diagnostics, Mumbai, India.

2.8. Assay of carbohydrate metabolism enzymes

The activities of the enzymes involving oxidation of NADH or reduction of NADP were determined spectrophotometrically on Cintra 5 fixed for 340 nm using 3 ml of assay buffer in a 1-cm cuvette at room temperature (28–30 °C). The enzyme activities of Lactate dehydrogenase (LDH, E.C.1.1.1.27), malate dehydrogenase (MDH, E.C.1.1.1.37), malic enzyme (ME, E.C.1.1.1.40), glucose-6-phosphate dehydrogenase (G6PDH, E.C.1.1.1.49), glucose-6-phosphatase (G6Pase, E.C.1.1.3.3) and fructose-1,6-bisphosphatase (FBPase, E.C.3.1.3.11) were assayed as described by Khandmuri et al., 2004. Hexokinase was estimated by the method of Crane and Sols, 1953 and the remaining glucose was measured by method of Nelson, 1944.
3. Results

3.1. Effect of dietary flaxseed oil on multiple NaAs dose induced alterations in serum and urine parameters

NaAs treatment to control rats resulted in significant increase in serum creatinine (Scr, +59.5%), blood urea nitrogen (BUN, +39.35%), cholesterol (+24.71%), phospholipids (PLP, +57.2%) but decrease in inorganic phosphate (–25.37%) and glucose (–24%) compared to control rats (Table 1). These changes were associated with massive glucosuria, phosphaturia, proteinuria and polyuria accompanied by decreased creatinine clearance (Table 2). In contrast FXO diet alone increased creatinine clearance and decreased polyuria, phosphaturia and proteinuria as compared to normal diet fed rats. Feeding of FXO-diet to NaAs administered (AsFXO group) rats resulted in amelioration of various NaAs elicited nephrotoxic alterations in serum and urine parameters. NaAs-induced increase in Scr, cholesterol and BUN were prevented by FXO supplementation diet.

Fig. 1. Experimental design. ND: normal diet, FXO: Flaxseed oil, NaAs: sodium arsenate, AsFXO: flaxseed oil + sodium arsenate treated, i.p.: intraperitoneal.

2.9. Assay of brush border membrane marker enzymes and lysosomal marker enzyme

The activities of brush border membrane marker enzymes alkaline phosphatase (ALP), leucine amino peptidase (LAP), γ-glutamyltransferase (GGTase) and lysosomal enzyme, acid phosphatase (ACPase) were determined as described by Farooq et al., 2004.

2.10. Assay of enzymes involved in free radical scavenging

Superoxide dismutase (SOD, E.C.1.15.1.1) was assayed by the method of Marklund and Marklund (1974), Catalase (E.C.1.11.1.6) and glutathione peroxidase (GSH-Px, E.C.1.11.1.9) activities were determined by the method of Giri et al. (1996) and Hohne and Gunzler (1984) respectively.

2.11. Thiobarbituric acid reactive substances (TBARS) and total-SH group estimation

TBARS and total SH groups were determined in the kidney homogenates. TBARS (products of LPO) were measured as malondialdehyde (MDA) equivalents (Ohkawa et al., 1979). Total SH groups were determined after reaction with 5,5′-dithiobisninobenzolic acid (Sedlak and Lindsay, 1968).

2.12. Statistical analysis

All data are expressed as mean ± SEM for at least 4–5 different preparations. Statistical evaluation was conducted by one-way ANOVA using origin 6.1 software followed by post hoc test (Student–Newman–Keuls and Dunnetts multiple comparison test). A probability level of p < 0.05 was selected as indicating statistical significance. Most of the changes between various groups were compared with control values for better understanding and clarity. However, specific differences and statistical significance between other groups were evaluated separately, e.g., NaAs vs. AsFXO.

3.2. Effect of dietary flaxseed oil (FXO) on multiple NaAs dose induced alterations in biomarker enzymes of BBM and lysosomes

To assess the structural integrity of certain organelles e.g., plasma membrane (BBM) and lysosomes, the effect of NaAs alone and in combination with FXO diet was determined on the biomarker enzymes of BBM and lysosomes in the homogenates of renal cortex and medulla and isolated BBM preparations from renal cortex.

3.3. Effect of NaAs alone and with FXO diet on biomarkers of BBM and lysosomes in the homogenates

The activities of alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGTase), leucine aminopeptidase (LAP) and acid phosphatase (ACPase) were determined under different experimental conditions in the homogenates of renal cortex and medulla (Table 3). NaAs treatment to control rats caused significant reduction in the specific activities of ALP (–26.33%), GGTase (–30.7%) and LAP (–34.6%) in cortical homogenate. The feeding of FXO diet prior to NaAs treatment prevented NaAs elicited decrease in BBM enzyme activities. As can be seen from the data, NaAs-induced a decrease in BBM enzyme activities which were similarly prevented by FXO diet. However, the activity of acid phosphatase (ACPase) was increased (+46.03%) by NaAs in cortical homogenates and FXO diet was able to prevent the increase in enzyme activity in a similar manner (Table 3). The activities of BBM enzymes similar to cortex, were also lowered in the medulla by NaAs administration. The consumption of FXO in combination with NaAs treatment resulted in the reversal of NaAs-induced decrease in GGTase (–30.37%) and LAP (–32.48%) in the medulla.

3.4. Effect of NaAs alone and with FXO diet on BBM markers in isolated BBMV

The effect of NaAs, FXO and their combination was further analyzed on the specific activities of BBM marker enzymes in BBMV isolated from renal cortex. The results summarized in Table 4 and Fig. 2, indicated that membrane preparations were several fold purified as reflected by the significant enrichment of ALP, GGTase and LAP activities in the BBM as compared to the homogenate (4–10-fold). The data summarized in Table 4, showed a similar activity pattern of BBM enzymes in BBM preparations as observed in the homogenates; however, the magnitude of effects under different experimental conditions became more apparent. The damaging effect of NaAs was clearly ameliorated by FXO, as NaAs-induced decrease in the activities of GGTase and LAP were not only prevented, rather the activities were found to be...
significant increase in AsFXO as compared to control group. Thus, it appears from the data that NaAs treatment to FXO fed rats significantly prevented NaAs-induced structural alterations in BBM integrity and alterations in the activities of its components.

3.5. Effect of FXO on NaAs-induced alterations on the enzymes of carbohydrate metabolism in rat renal tissues

The effect of NaAs, FXO and AsFXO was determined on the activities of various enzymes of carbohydrate metabolism in the kidney (Table 5). Activity of hexokinase was increased both in the renal cortex (+19%) and medulla (+18.8%) upon exposure to NaAs. Moreover, the activity of LDH (a marker of anaerobic glycolysis) was profoundly increased both in the renal cortex (+65%) and medulla (+51.3%). The activities of HK, LDH and MDH were lowered in NaAs- and FXO-treated groups compared to control. The activity of MDH was reduced to 4.84% and 2.44% in NaAs- and FXO-treated groups, respectively. The activity of LDH was reduced to 10.6% and 4.42% in NaAs- and FXO-treated groups, respectively. The activity of HK was reduced to 14.26% and 8.57% in NaAs- and FXO-treated groups, respectively.

### Table 1
Effect of FXO on serum parameters with/without NaAs treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Creatinine (mg/dl)</th>
<th>BUN (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>Phospholipid (mg/dl)</th>
<th>Phosphate (µmol/ml)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.818 ± 0.08</td>
<td>14.825 ± 1.905</td>
<td>171.77 ± 12.89</td>
<td>123.28 ± 4.39</td>
<td>4.69 ± 0.25</td>
<td>40.6 ± 0.405</td>
</tr>
<tr>
<td>NaAs (+32.25%)</td>
<td>1.305 ± 0.057</td>
<td>20.66 ± 0.747</td>
<td>214.22 ± 5.29</td>
<td>193.8 ± 5.24</td>
<td>3.5 ± 0.046</td>
<td>30.93 ± 3.1</td>
</tr>
<tr>
<td>NaAs (+39.35%)</td>
<td>(+24.18%)</td>
<td>(+8.8%)</td>
<td>(+4.84%)</td>
<td>(+19.3%)</td>
<td>(+7.25%)</td>
<td>(+24%)</td>
</tr>
<tr>
<td>AsFXO (+59.5%)</td>
<td>1.617 ± 0.027</td>
<td>14.59 ± 0.631</td>
<td>174.94 ± 11.03</td>
<td>147.12 ± 1.98</td>
<td>4.35 ± 0.149</td>
<td>41.79 ± 1.76</td>
</tr>
<tr>
<td>AsFXO (+70.96%)</td>
<td>(+9.16%)</td>
<td>(+115.3%)</td>
<td>(+8.5%)</td>
<td>(+19.34%)</td>
<td>(+7.25%)</td>
<td>(+2.93%)</td>
</tr>
<tr>
<td>FXO (+5.74%)</td>
<td>0.89 ± 0.09</td>
<td>11.24 ± 0.47</td>
<td>147 ± 9.02</td>
<td>109.31 ± 1.98</td>
<td>5.12 ± 0.47</td>
<td>45.16 ± 1.02</td>
</tr>
<tr>
<td>FXO (+22.22%)</td>
<td>(+26.33%)</td>
<td>(+7.7%)</td>
<td>(+22.22%)</td>
<td>(+46.03%)</td>
<td>(+46.03%)</td>
<td>(+46.03%)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for five different preparations. Values in parentheses represent percent change from control.

NaAs: sodium arsenate treated; AsFXO: flaxseed oil + sodium arsenate treated; and FXO: flaxseed oil diet.

### Table 2
Effect of flaxseed (FXO) on urine parameters of rats with/without NaAs treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Urine Flow Rate (UFR) (ml/day)</th>
<th>Creatinine clearance (ml/min/100 g bwt.)</th>
<th>Phosphate (µmol/ml)</th>
<th>Protein (mg/mmmols creatinine)</th>
<th>Glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.2 ± 1.25</td>
<td>0.42 ± 0.02</td>
<td>0.652 ± 0.008</td>
<td>2.19 ± 0.034</td>
<td>19.66 ± 1.25</td>
</tr>
<tr>
<td>NaAs (+30.7%)</td>
<td>10.6 ± 1.33</td>
<td>(+8.8%)</td>
<td>(+41.87%)</td>
<td>(+85.84%)</td>
<td>(+168.76%)</td>
</tr>
<tr>
<td>AsFXO (+32.25%)</td>
<td>8.2 ± 1.28</td>
<td>(+38.09%)</td>
<td>(+8.89%)</td>
<td>(+26.48%)</td>
<td>(+51.32%)</td>
</tr>
<tr>
<td>FXO (+39.35%)</td>
<td>6.5 ± 1.12</td>
<td>(+38.09%)</td>
<td>(+1.3%)</td>
<td>(+3.0%)</td>
<td>(+14.08%)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for five different preparations. Values in parentheses represent percent change from control.

Significantly different from control at p < 0.05 by one way ANOVA.

### Table 3
Effect of flaxseed oil on biomarkers of BBM and lysosomes in homogenates of (a) cortex and (b) medulla with/without NaAs treatment.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Groups</th>
<th>ALP (µmol/mg protein/h)</th>
<th>GGTase (µmol/mg protein/h)</th>
<th>LAP (µmol/mg protein/h)</th>
<th>ACPLase (µmol/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Cortex</td>
<td>Control</td>
<td>8.47 ± 0.54</td>
<td>16.64 ± 1.67</td>
<td>10.75 ± 1.01</td>
<td>3.15 ± 0.95</td>
</tr>
<tr>
<td>NaAs (+26.33%)</td>
<td>6.24 ± 0.12</td>
<td>(+26.33%)</td>
<td>(−30.7%)</td>
<td>(−34.6%)</td>
<td>(+46.03%)</td>
</tr>
<tr>
<td>AsFXO (+7.3%)</td>
<td>7.85 ± 0.74</td>
<td>(+26.33%)</td>
<td>(+2.22%)</td>
<td>(+7.7%)</td>
<td>(+3.17%)</td>
</tr>
<tr>
<td>FXO (+14.4%)</td>
<td>9.69 ± 0.75</td>
<td>(+26.33%)</td>
<td>(+9.99%)</td>
<td>(+29.86%)</td>
<td>(−9.21%)</td>
</tr>
<tr>
<td>(b) Medulla</td>
<td>Control</td>
<td>5.08 ± 0.55</td>
<td>18.27 ± 1.61</td>
<td>21.625 ± 2.22</td>
<td>4.93 ± 0.28</td>
</tr>
<tr>
<td>NaAs (+20.66%)</td>
<td>4.03 ± 0.94</td>
<td>(+20.66%)</td>
<td>(+30.7%)</td>
<td>(+32.48%)</td>
<td>(+16.43%)</td>
</tr>
<tr>
<td>AsFXO (+4.92%)</td>
<td>4.83 ± 0.73</td>
<td>(+4.92%)</td>
<td>(+7.69%)</td>
<td>(+14.19%)</td>
<td>(+4.6%)</td>
</tr>
<tr>
<td>FXO (+3.74%)</td>
<td>5.27 ± 0.61</td>
<td>(+3.74%)</td>
<td>(+22.22%)</td>
<td>(+22.22%)</td>
<td>(−4.6%)</td>
</tr>
</tbody>
</table>

Results (specific activity expressed as µmolues/mg protein/h) are Mean ± SEM for five different preparations.

Values in parentheses represent percent change from control.

Significantly different from control at p < 0.05 by one way ANOVA.

Significantly different from NaAs at p < 0.05 by one way ANOVA.
crease in superoxide dismutase (SOD, and (damage to renal tissues.
marked protection by FXO diet against NaAs induced oxidative
cant decline in lipid peroxidation both in cortex and medulla albeit
prevented the decline in SOD, GSH-Px and catalase activities. More-
the antioxidant parameters. NaAs administration to FXO fed rats
administration. However, FXO consumption significantly enhanced
GSH-Px (MDA) levels was significantly enhanced in the cortex (+89%) and
medulla (+79%), whereas total-SH declined in these tissues (−32.03%). The activity of ME, however was increased significantly
by NaAs treatment both in the renal cortex (+99%) and in renal me-
tive anabolic reactions. NaAs treatment to control rats significantly
shunt) and malic enzyme (ME), source of NADPH needed in reduc-
G6Pase and FBPase activities both in the renal cortex and medulla.
NaAs caused marked reduction in G6Pase and FBPase alterations in antioxidant enzymes both in cortex and medulla, albeit dif-
ters. NaAs enhanced lipid peroxidation (LPO) and significantly
increased risk of cancer and cardiovascular diseases in humans
(ATSDR, 1991). Arsenic compounds have been shown to cause se-
vere toxic effects in almost all the target organs including kidney
(Peraza et al., 2006; Mandal and Suzuki, 2002). The toxic effects of
arsenic are attributed to its ability to induce formation of ROS (Kok-
ilavani et al., 2005; Roy et al., 2009). Many different substances
including chelating agents (Gupta et al., 2005) have been investi-
gated for their protective potential in arsenic-induced toxicity.
However, most of them have not yet been proved safe for clinical
application. Recently, the use of naturally occurring antioxidant die-
tary substances for prevention of toxic effects of various toxicants and
environmental agents including metals/metalloids is gaining interest
(Priyamvada et al., 2010). Flaxseed oil (FXO) enriched in ω-3 PUFA (ALA) and phytoestrogenic lignans provide one such die-
tary source of biologically active components that has been shown
to be co-preventive and co-therapeutic in a wide variety of ailments
(Burde and Calder, 2005; Newairy and Abdou, 2009; Sinclair et al.,
2000). Dietary FXO consumption has been shown to ameliorate drug
(Naqshbandi et al., 2012) induced as well as lead acetate in-
duced toxicity (Rizwan et al., 2013). However, the efficacy of FXO against NaAs nephrotoxicity has not yet been explored.

3.6. Effect of dietary flaxseed oil (FXO) on multiple NaAs dose induced alterations in antioxidant defense parameters in renal cortex and medulla

To ascertain the role of antioxidant system in NaAs-induced tox-
icity, the effect of NaAs was observed on oxidative stress param-
ters. NaAs enhanced lipid peroxidation (LPO) and significantly
altered antioxidant enzymes both in cortex and medulla, albeit dif-
Tabelle 4

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Effect of FXO on activities of biomarker enzymes of BBM in cortical BBMV with/without NaAs treatment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>ALP (µmol/mg protein/h)</td>
</tr>
<tr>
<td>Control</td>
<td>57.76 ± 3.37</td>
</tr>
<tr>
<td>NaAs</td>
<td>25.63 ± 0.73</td>
</tr>
<tr>
<td>(−55.63%)</td>
<td>(−43.89%)</td>
</tr>
<tr>
<td>AsFXO</td>
<td>42.67 ± 2.8</td>
</tr>
<tr>
<td>(−26.12%)</td>
<td>(−9.0%)</td>
</tr>
<tr>
<td>FXO</td>
<td>69.28 ± 3.23</td>
</tr>
<tr>
<td>(+19.94%)</td>
<td>(+19.66%)</td>
</tr>
</tbody>
</table>

Results (specific activity expressed as µmoles/mg protein/h) are Mean ± SEM for five different preparations. Values in parentheses represent percent change from control.

4. Discussion

Arsenic is a well known environmental toxic associated with an increased risk of cancer and cardiovascular diseases in humans (ATSDR, 1991). Arsenic compounds have been shown to cause se-
vere toxic effects in almost all the target organs including kidney
(Peraza et al., 2006; Mandal and Suzuki, 2002). The toxic effects of
arsenic are attributed to its ability to induce formation of ROS (Kok-
ilavani et al., 2005; Roy et al., 2009). Many different substances
including chelating agents (Gupta et al., 2005) have been investi-
gated for their protective potential in arsenic-induced toxicity.
However, most of them have not yet been proved safe for clinical
application. Recently, the use of naturally occurring antioxidant die-
tary substances for prevention of toxic effects of various toxicants and
environmental agents including metals/metalloids is gaining interest
(Priyamvada et al., 2010). Flaxseed oil (FXO) enriched in ω-3 PUFA (ALA) and phytoestrogenic lignans provide one such die-
tary source of biologically active components that has been shown
to be co-preventive and co-therapeutic in a wide variety of ailments
(Burde and Calder, 2005; Newairy and Abdou, 2009; Sinclair et al.,
2000). Dietary FXO consumption has been shown to ameliorate drug
(Naqshbandi et al., 2012) induced as well as lead acetate in-
duced toxicity (Rizwan et al., 2013). However, the efficacy of FXO against NaAs nephrotoxicity has not yet been explored.

Multiple injections of NaAs were found to cause marked renal dysfunction as evident by increased serum creatinine (Scr) and BUN, diagnostic indicators of nephrotoxicity, accompanied by massive polyuria, proteinuria, glucosuria, phosphaturia and decreased creatinine clearance. These changes were associated with increased serum cholesterol and phospholipids but decreased serum Pi and glucose. Feeding of FXO enriched diet prior to and along with NaAs administration prevented NaAs-induced alterations in various serum/urine parameters. FXO consumption significantly lowered NaAs-elicited increased levels of Scr and BUN. Serum glucose and phosphate were also improved by dietary FXO supplementation.

Since the brush border membrane (BBM) lining the epithelial cells of the renal proximal tubule has been shown as the major site of metal-induced renal injury (Banday et al., 2008), the effect of NaAs was determined on the activities of BBM enzymes to assess the structural and functional damage caused by NaAs administration. The activities of BBM enzymes were significantly decreased in cortical and medullary homogenates after NaAs administration. However, the activities were decreased to greater extent in BBMV than in the respective cortical homogenates. Thus, the membrane bound enzymes appear to be more sensitive to NaAs than the sol-
uble enzymes. The decrease in BBM enzyme activities could be due to the oxidative modification and consequent inactivation of
(Fig. 3A) and FXO (Fig. 3D) treated rats showed normal architecture of the kidney.
enzymes by NaAs generated free radicals and ROS. Increased LPO, which affects membrane structure and function, could also have resulted in a decrease in the activities of these enzymes. There could have been leakage or loss of BBM enzymes into the tubular lumen following ROS-induced damage to the epithelial cells, especially to the membrane and these enzymes later appear in the urine as demonstrated previously for other toxicants (Banday et al., 2008) and reflected by observed proteinuria. In contrast to NaAs, FXO consumption alone, however, caused significant increase in the activities of BBM enzymes in homogenate as well as in BBMV, indicating an overall improvement in renal BBM integrity as shown earlier (Naqshbandi et al., 2012; Priyamvada et al., 2010). FXO consumption in combination with NaAs treatment prevented/retarded NaAs-induced decrease in BBM enzyme activities in BBMV and homogenate. The ameliorating effect of FXO might be attributed to its intrinsic antioxidant and/or free radical scavenging properties associated with its constituent bioactive components viz. ω-3 PUFAs (Sinclair et al., 2000; Burdge and Calder, 2005) and lignans (Newairy and Abdou, 2009). The activity of lysosomal enzyme, ACPase was significantly increased in the cortex by NaAs treatment. Morphologically increase in the number of lysosomes by NaAs treatment has been shown previously (Brown et al., 1976). The present results indicate that renal BBM and lysosomes were severely damaged by NaAs, however, FXO consumption prevented NaAs effects by lessening the damage caused or by increasing the regeneration process or both (Naqshbandi et al., 2012).

Since the major function of kidney is to reabsorb important ions and molecules, and that in turn depends on the structural integrity of the renal proximal tubule and its BBM and also on the available energy as ATP which is supplied by various metabolic pathways, it is imperative that any alterations in metabolic activities caused by toxic insult or otherwise modified would affect the renal functional capabilities. To assess the functional aspects, the activities of various enzymes of carbohydrate metabolism were examined in the renal tissue homogenates in different experimental groups. The activities of various enzymes involved in glycolysis, TCA cycle, gluconeogenesis and HMP-shunt pathway were differentially altered by NaAs treatment and/or by FXO consumption. NaAs caused significant increase in LDH (glycolysis) and decrease in MDH (TCA cycle) activity both in the renal cortex and medulla, which was associated with simultaneous increase in hexokinase activity in the renal tissues. Although the actual rates of glycolysis or TCA cycle were not determined, marked decrease in MDH activity might be due to NaAs-induced damage to mitochondria (Brown et al., 1976; Pulido and Parrish, 2003). A marked increase in LDH and to some extent hexokinase activity with simultaneous decline in TCA cycle enzyme, MDH appears to be an adaptive cellular effect in energy metabolism from aerobic metabolism alternatively to anaerobic glycolysis due to NaAs induced mitochondrial dysfunction. The observed effects of NaAs on the functional capabilities of proximal tubules were found to be consistent with histopathological findings where severe damage to renal corpuscles and renal tubules by NaAs leads to congestion of glomeruli, dilatation of renal tubules, blebbing from the apical surface of the tubular lining. However, FXO could prevent the changes and could also maintain the structure almost similar to that of control.

NaAs also differentially altered the activities of enzymes of gluconeogenesis and HMP-shunt pathway. The activities of G6Pase and FBPase (gluconeogenesis) and G6PDH (HMP shunt) were profoundly decreased in both cortex and medulla albeit to different extent. The decreased activities of gluconeogenic enzymes may be the consequence of decreased MDH activity. In arsenic exposed animals the reduced production of oxaloacetate from malate seems to affect both TCA cycle and gluconeogenesis. The oxidative conversion of glucose or glucose-6-phosphate to 6 phosphogluconate by G6PDH via HMP shunt pathway was also lowered in part due to mitochondrial dysfunction or by inhibition of functional SH groups in G6PDH by NaAs (Stohs and Bagchi, 1993). However, the activity of NADP malic enzyme (ME) variably increased in the renal tissues. The present data indicate that NaAs caused differential effect on different enzymes of the carbohydrate metabolism. However, FXO administration to NaAs-treated rats resulted in an overall improvement of carbohydrate metabolism as evident by
and then to molecular oxygen and water. Another enzyme is
higher activities of LDH, MDH and gluconeogenic enzymes in AsF-
XO compared to NaAs alone or control groups. This could be attributed
to the fact that FXO might have lowered the number of damaged mitochondria or affected macromolecules or increased the number of normally active organelles or macromolecules.

The underlying mechanism by which arsenic causes nephro-
toxicity is not well understood. However, there are reports suggesting that ROS are important mediators of arsenic nephropathy (Sinha et al., 2008). Metabolism of arsenic in the cells leads to the generation of ROS like superoxide anion (O₂⁻), hydroxyl radical (·OH), hydrogen peroxide (H₂O₂), singlet oxygen (¹O₂), and persulphyl radicals, which are responsible for various toxic effects (Liu et al., 2000) including oxidative damage to cellular macromolecules like DNA, proteins and lipids by disturbance of antioxidant defence system (Imlay, 1998; Muthumani and Prabu, 2012). A major cellular defense against ROS is provided by SOD and catalase, which together convert superoxide radicals first to H₂O₂ and then to molecular oxygen and water. Another enzyme viz. GSH-Px utilizes thiol-reducing power of glutathione to reduce oxidized lipids and protein targets of ROS. Arsenic has been shown to enhance lipid peroxidation (LPO), an indicator of tissue injury and to deplete GSH and protein thios (Valko et al., 2005). The present results show that NaAs administration to control rats caused severe damage to renal tissues mediated by ROS as apparent by decrease in the antioxidant enzymes (SOD, catalase and GSH-Px) and total-SH content accompanied with increased lipid peroxidation. The membrane polyunsaturated fatty acids (PUFA) are highly susceptible to free radical-induced oxidative damage. Interaction of reactive oxygen species with PUFA initiates the self-propagating lipid peroxidation reactions (Pepicelli et al., 2005) resulting in impaired membrane function and integrity as well as inactivation of several membrane bound enzymes (Halliwell and Gutteridge, 1999). We observed that NaAs treatment led to excessive lipid peroxidation with overwhelmingly higher levels of malondialdehyde in the renal tissues. Thus, NaAs-induced lipid peroxidation leads to the degradation of phospholipids and finally results in the renal cellular deterioration. The decline in total SH content of the tissue caused by treatment with

Table 5

Effect of flaxseed oil (FXO) on carbohydrate metabolic enzymes with/without NaAs treatment in cortical and medullary homogenates.

<table>
<thead>
<tr>
<th>Enzyme Groups</th>
<th>LDH (µmol/mg protein/h)</th>
<th>MDH (µmol/mg protein/h)</th>
<th>HK (µmol/mg protein/h)</th>
<th>G6Pase (µmol/mg protein/h)</th>
<th>FBPase (µmol/mg protein/h)</th>
<th>ME (µmol/mg protein/h)</th>
<th>GDPDH (µmol/mg protein/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14.99 ± 0.69</td>
<td>12.26 ± 0.92</td>
<td>3.48 ± 0.24</td>
<td>1.29 ± 0.21</td>
<td>2.59 ± 0.24</td>
<td>0.185 ± 0.024</td>
<td>0.251 ± 0.02</td>
</tr>
<tr>
<td>NaAs</td>
<td>24.77 ± 1.13</td>
<td>6.95 ± 0.67</td>
<td>4.16 ± 0.21</td>
<td>0.96 ± 0.13</td>
<td>1.78 ± 0.11</td>
<td>0.369 ± 0.06</td>
<td>0.144 ± 0.01</td>
</tr>
<tr>
<td>AsFXO</td>
<td>18.42 ± 1.29</td>
<td>9.43 ± 0.29</td>
<td>3.76 ± 0.38</td>
<td>1.43 ± 0.18</td>
<td>2.32 ± 0.22</td>
<td>0.148 ± 0.004</td>
<td>0.237 ± 0.02</td>
</tr>
<tr>
<td>FXO</td>
<td>13.8 ± 0.52</td>
<td>10.85 ± 0.3</td>
<td>2.91 ± 0.28</td>
<td>1.4 ± 0.22</td>
<td>2.66 ± 0.25</td>
<td>0.196 ± 0.006</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>Medulla</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.02 ± 0.51</td>
<td>2.59 ± 0.23</td>
<td>2.13 ± 0.09</td>
<td>1.19 ± 0.04</td>
<td>1.17 ± 0.04</td>
<td>0.143 ± 0.007</td>
<td>0.128 ± 0.01</td>
</tr>
<tr>
<td>NaAs</td>
<td>17.27 ± 0.97</td>
<td>1.26 ± 0.03</td>
<td>2.53 ± 0.113</td>
<td>0.947 ± 0.07</td>
<td>0.873 ± 0.077</td>
<td>0.293 ± 0.02</td>
<td>0.087 ± 0.01</td>
</tr>
<tr>
<td>AsFXO</td>
<td>12.25 ± 0.82</td>
<td>2.24 ± 0.58</td>
<td>2.06 ± 0.13</td>
<td>1.16 ± 0.22</td>
<td>1.06 ± 0.08</td>
<td>0.198 ± 0.006</td>
<td>0.118 ± 0.006</td>
</tr>
<tr>
<td>FXO</td>
<td>7.54 ± 0.23</td>
<td>2.1 ± 0.27</td>
<td>1.91 ± 0.15</td>
<td>1.29 ± 0.10</td>
<td>1.14 ± 0.11</td>
<td>0.171 ± 0.03</td>
<td>0.143 ± 0.02</td>
</tr>
</tbody>
</table>

Results (specific activity expressed as µmoles/mg protein/h) are Mean ± SEM for five different preparations.

Values in parentheses represent percent change from control.

* Significantly different from control at p < 0.05 by one way ANOVA.

<sup>1</sup> Significantly different from NaAs at p < 0.05 by one way ANOVA.

Table 6

Effect of flaxseed oil (FXO) on enzymatic and non-enzymatic antioxidant parameters in homogenates of (a) cortex and (b) medulla with/without NaAs treatment.

<table>
<thead>
<tr>
<th>Parameters Groups</th>
<th>Lipid Peroxidation (nmols/gm tissue)</th>
<th>Total SH (µmoles/gm tissue)</th>
<th>SOD (Units/mg protein)</th>
<th>Catalase (µmoles/mg protein/min)</th>
<th>GSH-Px (µmoles/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>78.07 ± 11.15</td>
<td>6.135 ± 0.545</td>
<td>213.55 ± 11.86</td>
<td>4.8 ± 0.02</td>
<td>0.092 ± 0.003</td>
</tr>
<tr>
<td>NaAs</td>
<td>147.88 ± 8.87 (†)</td>
<td>4.796 ± 0.21</td>
<td>149.33 ± 4.75</td>
<td>3.37 ± 0.02 (†)</td>
<td>0.054 ± 0.0025 (†)</td>
</tr>
<tr>
<td>AsFXO</td>
<td>92.88 ± 9.66 (††)</td>
<td>5.47 ± 0.67</td>
<td>213.08 ± 10.47</td>
<td>4.22 ± 0.28 (††)</td>
<td>0.071 ± 0.003 (††)</td>
</tr>
<tr>
<td>FXO</td>
<td>65.38 ± 8.46 (†)</td>
<td>6.105 ± 0.035</td>
<td>252.72 ± 15.43</td>
<td>6.26 ± 0.03 (‡)</td>
<td>0.12 ± 0.005 (‡)</td>
</tr>
<tr>
<td>(b) Medulla</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>53.71 ± 8.24</td>
<td>4.55 ± 0.14</td>
<td>207.75 ± 4.24</td>
<td>5.33 ± 0.33 (‡)</td>
<td>0.045 ± 0.005 (‡)</td>
</tr>
<tr>
<td>NaAs</td>
<td>95.95 ± 3.76 (†)</td>
<td>3.9 ± 0.21</td>
<td>179.56 ± 7.08</td>
<td>4.82 ± 0.47 (‡)</td>
<td>0.021 ± 0.002 (‡)</td>
</tr>
<tr>
<td>AsFXO</td>
<td>53.08 ± 4.71 (††)</td>
<td>4.28 ± 0.47</td>
<td>191.06 ± 4.55</td>
<td>6.00 ± 1.62 (‡†)</td>
<td>0.033 ± 0.004 (‡†)</td>
</tr>
<tr>
<td>FXO</td>
<td>39.1 ± 3.6 (†††)</td>
<td>4.63 ± 0.2</td>
<td>211.98 ± 2.98</td>
<td>6.15 ± 0.714 (†††)</td>
<td>0.039 ± 0.005 (†††)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for five different preparations.

Values in parentheses represent percent change from control.

† Significantly different from control at p < 0.05 by one way ANOVA.

Significantly different from NaAs at p < 0.05 by one way ANOVA.
NaAs can be explained by the fact that thiol groups are quite reactive towards metal ions and are also easily oxidizable by ROS (Valko et al., 2005). Diminished SH content has been reported upon exposure of tissues to heavy metals or drugs (Valko et al., 2005). When FXO was administrated to NaAs treated rats the decline in SH content was prevented. This may be attributed to the possibility of arsenic getting chelated to the hydroxyl groups of lignans, which are the bioactive constituents of FXO. However, the NaAs-induced changes in malondialdehyde and antioxidant enzymes were attenuated by dietary supplementation of FXO which could have prevented/reduced the NaAs-induced generation of free radicals and ROS. The protection against NaAs effect by FXO can be attributed to its intrinsic biochemical and natural antioxidant properties. Thus, it appears that FXO enriched in ω-3 fatty acids and lignans enhanced resistance to free radical attack generated by NaAs administration.

To conclude the present investigation clearly demonstrate that NaAs produces severe nephrotoxicity and causes profound damage to plasma membranes of the renal proximal tubules. The enzymes of oxidative carbohydrate metabolism and gluconeogenesis; BBM and antioxidant defense mechanism appeared to be severely affected by NaAs treatment. In contrast, FXO, a major source of omega-3 fatty acids and phytoestrogenic lignans appears to improve membrane organization/integrity and functions. FXO might have accelerated the repair and/or regeneration of injured organelles e.g., mitochondria and plasmamembrane viz BBM as evident by increased activities of TCA cycle and BBM enzymes and as observed by histopathological studies. Most importantly, by strengthening endogenous antioxidant defense, FXO counter-acted free radical mediated arsenic toxicity.

Conflict of Interest

The authors declare that there are no conflicts of interest in this work.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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