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Oleuropein ameliorates arsenic induced oxidative stress in mice

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ABSTRACT

The objective of this study is to investigate the potential preventive effect of oleuropein in an experimental arsenic toxicity in mice. For this purpose, mice were exposed to 5 mg/kg/day sodium arsenite ($NaAsO_2$) in drinking water and treated with 30 mg/kg/day oleuropein for 15 days. At the end of the experiment, animals were sacrificed and selected organs were processed for biochemical and histopahtological investigations. Blood, liver, kidney and brain malondialdehyde (MDA) and nitric oxide (NO) levels were determined by colorimetric methods. Protein carbonyl content is measured by a commercial kit. Liver morphology and immunoreactivity for inducible NOS (iNOS) and endothelial NOS (eNOS) was evaluated microscopically. Level of NO was determined to decrease in blood and tissues whereas MDA increased in arsenic given mice. Tissue protein carbonyl content also increased in this group. Immunoreactivity for iNOS and eNOS was noted to increase with arsenic treatment. Oleuropein treatment had significant effects in normalizing the MDA and NO levels as well as protein carbonyl content. Immunohistochemical staining also showed reduction of the expression of iNOS and eNOS in liver. The results indicate that oleuropein ameliorates oxidative tissue damage by scavenging free radicals.

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1. Introduction

Arsenic (As) is one of the most important toxic heavy metal ubiquitously found in natural environment. Arsenic can be found in water, soil and air inorganic forms as well as organic forms. The most common source of arsenic exposure is through consumption of contaminated drinking water [1,2]. As^{+3} (trivalent) and As^{+5} (pentavalent) are forms of inorganic arsenic mostly found in water. The trivalent form of arsenic is more toxic than the pentavalent form [3,4].

It has been known for a long time that people are prone to cancers, black foot diseases, diabetes mellitus (type II) [5,6], cardiovascular diseases [7], hypertension [8] and neurological diseases such as Alzheimer and Parkinson [9,10] exposed to high level of arsenic.

Arsenic causes toxicity in human and animal cells by development of oxidative stress [11,12]. Oxidative stress in arsenic toxicity is mediated by its oxidizing effect on metals such as iron (Fe) or reaction with antioxidants [13]. Arsenic also increases consumption of oxygen by the cells which increase reactive oxygen species (ROS) production and oxidative stress [14,15]. Indeed damage to proteins is more important than lipids in situation of oxidative stress [14].

Olive oil as a source of fat is one of the main nutrients in the conventional Mediterranean diet. The most bioactive component in olive oil is oleuropein. Oleuropein act as a free radical scavenger due to its strong antioxidant activity [16]. Most epidemiological studies revealed that in Mediterranean where the olive oil used abundantly widespread the incidence of certain cancers such as breast and colon and coronary heart disease is lower [17]. Because of the strong antioxidant capacity of oleuropein and the known mechanism of arsenic toxicity, it was aimed in this study to investigate the potential preventive effect of oleuropein in an experimental arsenic toxicity model by means of biochemistry and histopathology.

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2. Materials and methods

2.1. Experimental design

The ethics approval for research was confirmed by Kafkas University Animal Care and Use Committee (Registration Number: 2012-08). All procedures were conducted in accordance with the 'Guide for Care and Use of Laboratory Animals' published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain.

Male Swiss albino mice used in this experiment were purchased from Laboratory Animal Experimental Units of Ataturk University, Turkey. The mice were housed at 25 ± 2 °C, with an average humidity 50 ± 5% and with a lighting schedule of 12 h light and 12 h dark. Total of 40 male *swiss albino* mice weighing 26–32 g at 10–11 weeks of age were randomly divided into 4 equal groups, 10 mice each, and kept in cages. No attempt was made to normalize different groups by body weight. Animals had free access to a standard pellet diet and water *ad libitum*. No experimental treatment was performed on Control group (C). Mice in arsenic group (As) received 5 mg/kg/day sodium arsenite ($NaAsO_2$) (Sigma Chemical Company) in drinking water. Arsenic plus oleuropein group (As + OLE) received 5 mg/kg/day sodium arsenite plus 30 mg/kg/day oleuropein in drinking water, and oleuropein group (OLE) had only 30 mg/kg/day oleuropein. All experimental treatments lasted 15 days.

2.2. Biochemical analysis

At the end of the experiment blood samples were collected into heparin tubes from the hearts via cardiac puncture under ether anesthesia for MDA and NO measurements. The blood samples were centrifuged (3000*g*, 4 °C) for 10 min, and the plasma were obtained and then kept at −25 °C until the analyses were carried out. At necropsy liver, kidney, and brain tissues were collected to determine tissue MDA, NO and protein carbonyl content concentrations. For this purpose, the tissues were rinsed with ice-cold 0.9% NaCl. Then, 1 g of tissue was homogenized in phosphate buffer (pH 7.4) in an ice bath and the homogenates were centrifuged (5000*g*, 4 °C) for 15 min.

MDA as an end product of lipid peroxidation concentrations were measured by the method of Yoshoiko et al. [18] based on the reaction between thiobarbituric acid and MDA. The end products were read at 535 nm.

NO levels were determined according to the method described by Miranda et al. [19] in that nitrate is reduced to nitrite by $VaCl_3$, and then in acidic environment nitrite was reacted with sulphanilamide to produce colored diazonium compound, which was read at 540 nm.

Protein carbonyl content is measured by a commercial spectrophotometric assay kit (Cell Biolabs, INC). Bovine serum albumin (BSA) standards or protein samples which are derivate with dinitrophenylhydrazine (DNPH) first. Proteins are then trichloroacetic acid (TCA) precipitated and free DNPH is removed by washing the protein pellet. After dissolving the protein pellet in guanidine hydrochloride (GuHCl), the absorbance of protein-hydrozone is measured at 375 nm. MDA, NO and protein carbonyl content levels were determined by using a spectrophotometer (Epoch, Biotech, USA).

2.3. Histopathology and immunohistochemistry

Pieces of liver tissue samples collected at necropsy were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Then, 5 μm thick sections were cut from the paraffin blocks, and were routinely processed for hematoxylin and eosin staining. The tissue sections were observed under a light microscope for evaluation of pathological changes.

iNOS and eNOS activities in liver tissues were assessed immunohistochemically. For this purpose, 4 μm thick sections were cut from the paraffin blocks, deparaffinized via conventional methods, and then washed in phosphate buffered saline (PBS, pH 7.4). Tissue endogenous peroxidase activity was blocked by 0.3% H_2O_2 for 20 min. Then, tissue antigen retrieval was accomplished by microwave treatment in 10 mM citrate buffer, pH 6.0 for 10 min. Following several PBS washes, the sections were incubated for 30 min at room temperature with non-immune goat serum (Zymed Laboratories Inc., Cat No: 85-9043A) to block non-specific antibody binding. The sections were treated with rabbit anti-iNOS antibody (Thermo Scientific, Cat No: RB-9242-PO) diluted 1:100 and rabbit anti-eNOS antibody (Thermo Scientific, Cat No: RB-9279-PO) diluted 1:50. Antibody incubations were performed in a humidified chamber at room temperature for 1 h. Biotinylated secondary antibody and then streptavidin peroxidase complex (Zymed Histostain- Plus Bulk Kit, cat. No. 85-9043) were applied on to the sections for 30 min each with several PBS washes between. Antibody binding was visualized by color development using 3,3-diaminobenzidine–H_2O_2. Finally, the sections were counterstained with Mayer's hematoxylin, washed with water, processed in an alcohol–xylene series, covered with a cover slip, and examined under a light microscope.

Table 1
Effects of oleuropein on MDA and NO levels (μmol/L) in blood. Values are expressed as mean ± SD. Values with different superscripts within the same column significantly differ from each other ($P < 0.01$).

	MDA	NO
Control	14.21 ± 0.24[a]	10.94 ± 0.25[a]
As	23.16 ± 0.88[b]	5.83 ± 0.63[b]
As + OLE	17.32 ± 0.63[c]	7.36 ± 0.32[c]
OLE	12.25 ± 0.57[d]	8.25 ± 0.74[d]

2.4. Statistical analysis

Statistical analyses of biochemical data were done by SPSS software (Windows version 20.0). The data were expressed as median (X) ± standard deviation (SD). Importance level of difference among the groups was determined by variance analysis (ANOVA) and Duncan multiple comparison test.

3. Results

3.1. Levels of MDA and NO

The results of blood MDA and NO levels were summarized in Table 1. The levels of MDA in blood were significantly higher in As group, compared to those of control group animals. Conversely blood MDA level in OLE group decreased compared to As and control groups. NO levels decreased significantly in mice treated $NaAsO_2$ compared to that of mice in control ($p < 0.01$).

Liver, kidney and brain MDA and NO levels were shown in Table 2. The kidney and brain tissue levels of MDA in As group were determined to be significantly higher than the control group ($p < 0.01$). Liver, kidney and brain NO levels were detected to be significantly lower in As group than the control. The brain MDA and NO levels in OLE group were noted to be significantly lower than the control ($p < 0.01$).

3.2. Protein carbonyl content

Protein carbonyl content levels are shown in Table 3. Treatment of arsenic significantly increased protein carbonyl content

Table 2
Effects of oleuropein on MDA and NO (μmol/L g wet tissue) in liver, kidney and brain. Values with different superscripts within the same column significantly differ from each other ($P < 0.01$).

Groups	Liver		Kidney		Brain	
	MDA	NO	MDA	NO	MDA	NO
Control	3.92 ± 0.23^a	5.27 ± 0.24^a	6.18 ± 0.18^a	8.18 ± 0.21^a	1.62 ± 0.13^a	6.62 ± 0.16^a
As	11.74 ± 0.42^b	2.74 ± 1.02^b	12.23 ± 0.21^b	5.23 ± 0.51^b	8.85 ± 0.22^b	2.85 ± 0.41^b
As + OLE	7.61 ± 0.35^c	3.64 ± 0.37^c	9.21 ± 0.42^c	6.21 ± 0.32^c	6.79 ± 0.11^c	4.79 ± 0.38^c
OLE	3.78 ± 0.29^a	4.96 ± 0.45^a	5.78 ± 0.26^a	8.17 ± 0.11^a	1.54 ± 0.11^d	6.14 ± 0.14^d

in liver, kidney and brain tissues ($p < 0.001$). However, oleuropein treatment allied the changes in protein carbonyl content, and hence restored protein oxidative damage significantly ($p < 0.001$).

3.3. Histopathology

Microscopic investigations of liver tissues in Control (Fig. 1a) and oleuropein (Fig. 1b) groups showed normal histomorphology. In As group, severe hydropic degeneration with occasional focal necrosis in 6 mice and moderate hydropic degeneration together with Kupffer cell proliferation and dissociation in remark cords in 4 cases (Fig. 1c) were determined. Compared to the As group, a significant reduction in the number and severity of degenerative and necrotic changes were detected in As + OLE. In this group, hydropic degeneration was recognized little in 5, moderate in 4, and severe only in 1 animal. Slight activation in Kupffer cells were also noted (Fig. 1d). Necrotic changes were detected only in one case.

3.4. iNOS and eNOS immunoreactivity

iNOS immunoreactivity was seen to be intracytoplasmic in hepatocytes in liver tissues. Generally little or no immunoreactivity was observed in Control (Fig. 2a) and oleuropein (Fig. 2b) groups. In Arsenic group, degenerate hepatocytes and activated Kupffer cells showed strong immunoreactivity for iNOS in their cytoplasm with no specific lobular localization (Fig. 2c). Oleuropein treatment in arsenic given mice showed iNOS reactivity also in degenerate cells and Kupffer cells (Fig. 2d), however the degree of immunostaining was less severe in this group compare to the arsenic only given group.

eNOS expression in livers of control (Fig. 3a) and oleuropein (Fig. 3b) groups were seen to be located in the cytoplasm of sinusoidal endothelial cells and few hepatocytes located especially close to the central vein. In the arsenic group, no immunostaining was observed in areas where necrotic changes were present. Immunoreactivity in this group was noted to be little in sinusoidal endothelial cells and strong in hepatocytes (Fig. 3c). In As + OLE group, reduced eNOS immunoreactivity in sinusoidal endothelial cells comparable to As only group was observed. Few hepatocytes also showed immunostaining in this group (Fig. 3d).

4. Discussion

Countless toxic substances around us can cause important cellular degenerations. The most efficient cellular defense mechanisms against these toxic compounds are the act of antioxidants that are either naturally present or taken into the body. Many enzymatic and non-enzymatic factors are known to contribute to cellular maintenance by scavenging and clearing ROS. Arsenic-induced toxic changes in several organs have been previously described and shown to be the results of induction of ROS and depletion of antioxidant defenses [20,21]. Therefore, the theory of increasing antioxidant capacity in cells to ameliorate the toxic effects caused by arsenic was investigated in the present study.

Oleuropein, which is a strong antioxidant agent, protects biological molecules and is a target for toxic compounds [21]. Its antioxidant properties are seen in response to both preventative and intervention effects. Oleuropein may prevent free radical formation by the means of both chelating metal ions that catalyzes ROS formation and inhibiting enzymes like lipoxygenases. Providing hydroxyl group to suppress ROS formation is the way of its action [22].

Arsenic toxicity causes an immediate increase in ROS production, which further mediated lipid peroxidation. Although ROS are short-lived molecules they can attack polyunsaturated fatty acids present in many membranes and therefore trigger lipid peroxidation within the cells. MDA is formed as a by-product of lipid peroxidation that has longer half-lives and is more potent to reach distant intra- and extra-cellular sites from where it is produced. MDA is produced during lipid peroxidation and hence amplifies oxidative stress [23]. MDA levels in serum, kidney, brain, and liver tissues were also detected to be significantly higher in the arsenic given mice in the present investigation. Moreover, oleuropein treatment in arsenic given mice was shown to decrease MDA levels significantly compared to the arsenic only given group [24]. This result indicates that oleuropein could help preventing lipid peroxidation in arsenic toxicity.

ROS may damage all types of biomolecules such as DNA, proteins and lipids. However, proteins are more vulnerable to oxidative damage on cells [25]. ROS giving rise to protein oxidation are radical species such as superoxide ($O^{\bullet}{}_2{}^-$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2{}^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroperoxyl ($HO_2{}^{\bullet}$), and nonradical species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$). Protein carbonyl content is accepted as the most commonly used marker for protein oxidation [26]. In the present study, the mean values of liver, kidney, and brain protein carbonyl content in arsenic only given group were significantly higher than the control group. This result complies with the results of a previous study where arsenic induced increased protein carbonyl content was detected in mice [27]. In the current investigation, oleuropein treatment was shown to decrease protein carbonyl content.

NO, which is a short-lived gas, has effects on virtually every physiological processes in various organs and tissues. NO is produced by a family of nitric oxide synthases (NOS). eNOS (endothelial NOS) and nNOS (neuronal NOS) is mediated in mammals by the calcium-calmodulin controlled isoenzymes. In immune responses, the inducible isoform (iNOS) is involved in the production of NO that acts in immune defenses. NO that is produced in endothelial cells regulates blood pressure and interacts between platelets and the vessel walls. Under certain conditions, eNOS and nNOS are

Table 3
Effect of oleuropein on protein carbonyl content (PCC) (nmol/mg g wet tissue) in liver, kidney and brain. Values with different superscripts within the same column significantly differ each other ($P < 0.001$).

Groups	Liver	Kidney	Brain
Control	8.66 ± 0.18^a	9.32 ± 0.11^a	7.27 ± 0.16^a
As	23.15 ± 0.55^b	18.23 ± 0.51^b	18.85 ± 0.41^b
As + OLE	16.64 ± 0.37^c	9.26 ± 0.45^c	13.79 ± 0.38^c
OLE	8.59 ± 0.15^a	8.27 ± 0.05^a	6.21 ± 0.14^d

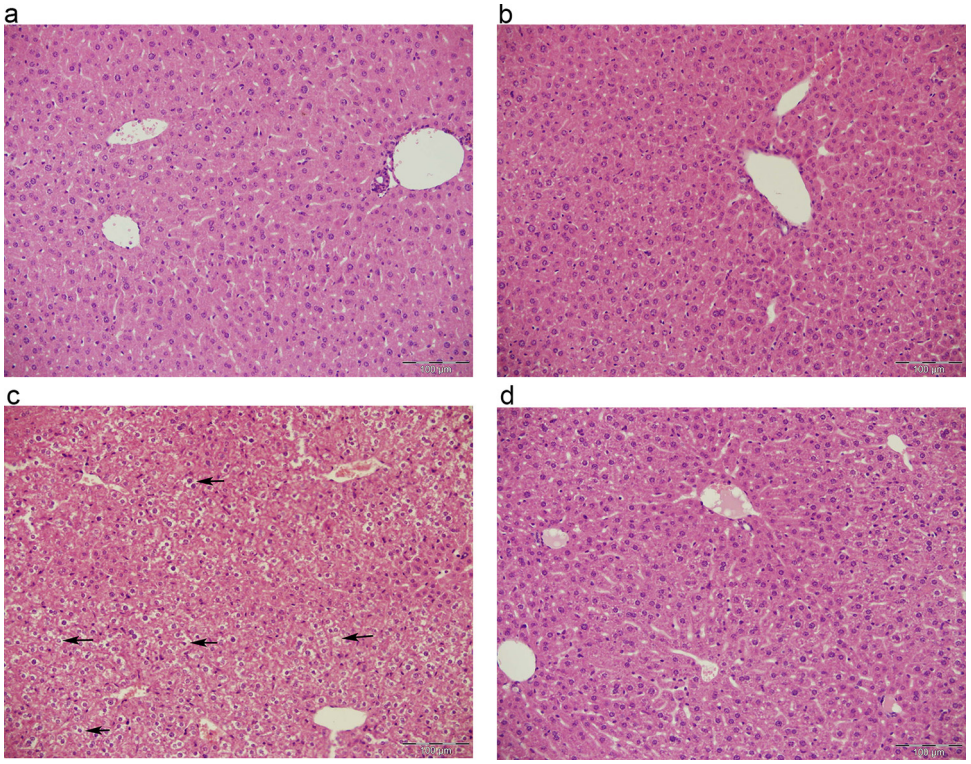


Fig. 1. Normal liver histomorphology in control (a) and oleuropein (b) groups. Severe hydropic degeneration (arrows), occasional necrosis and activation in Kupffer cells in arsenic group (c). Midzonal moderate degeneration in arsenic plus oleuropein group (d). HE.

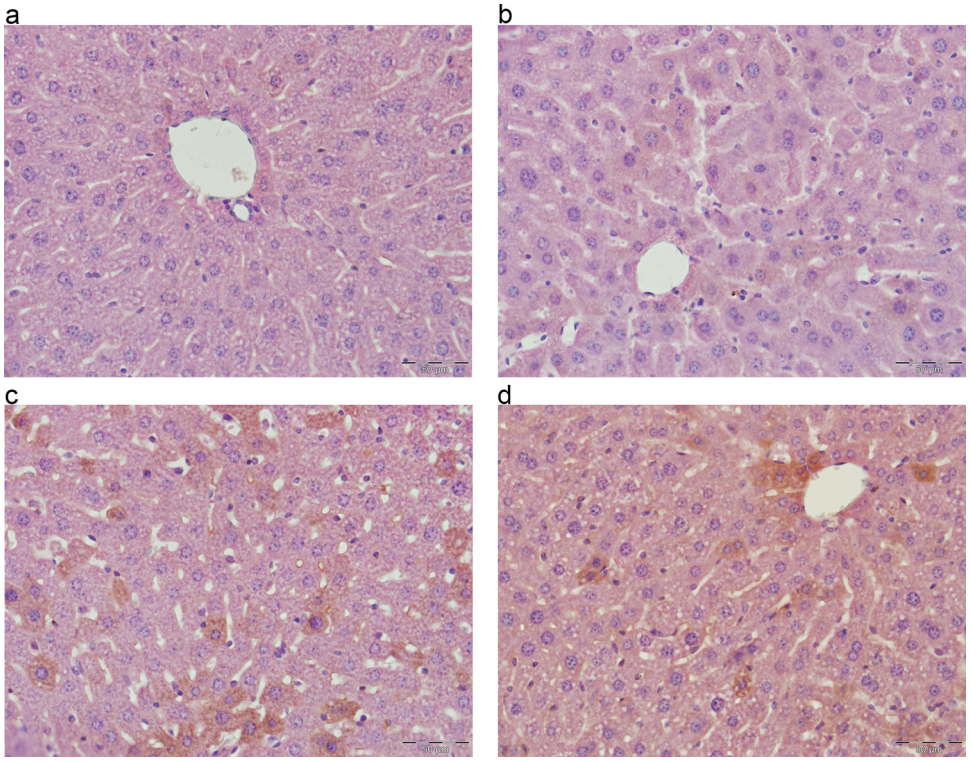


Fig. 2. Immunohistochemical reactivity for inducible nitric oxide synthase (iNOS). Little or no immunoreactivity in control (a) and oleuropein (b) groups. Strong immunoreactivity in hepatocytes for iNOS in arsenic group (c). Moderate immunoreactivity in arsenic plus oleuropein group (d).

called as constitutive nitric oxide synthases (cNOS). L-arginine as a precursor of NO and cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4) are the key biomolecules in NO production. BH_4 plays key role in the control of NO production [28,29].

In the present study, significant decreases in NO levels were observed in blood, liver, kidney and brain tissue in arsenic given group compared to the others. This result complies with a previous study in that the levels of nitrite and nitrate, the stable metabo-

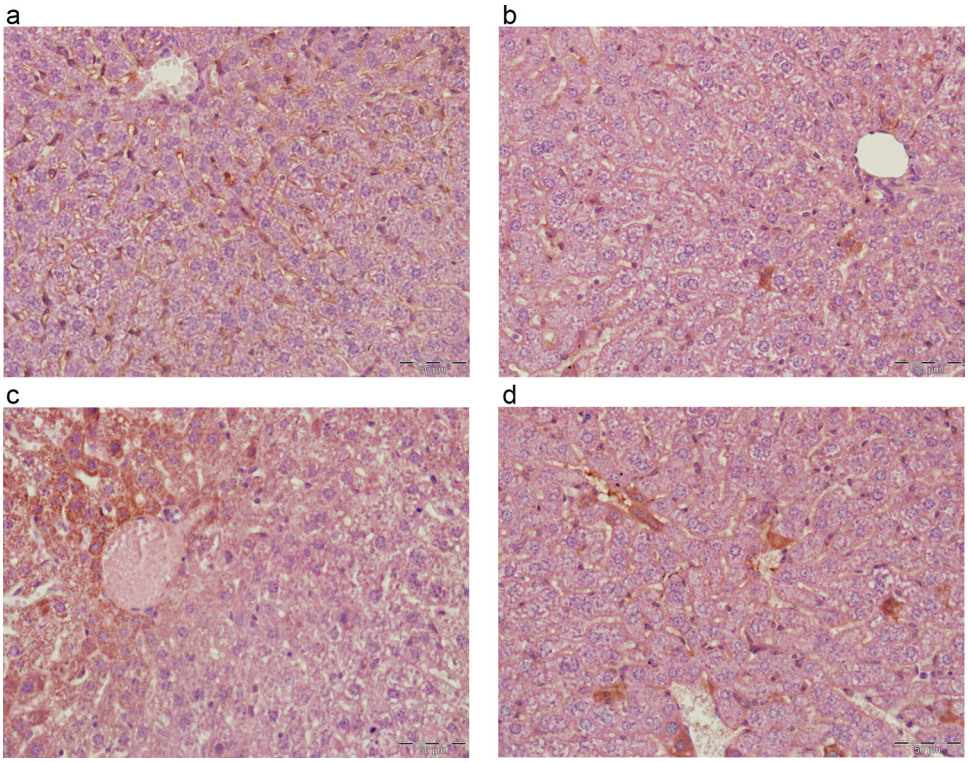


Fig. 3. Immunohistochemical reactivity for endothelial nitric oxide synthase (eNOS). Strong immunoreactivity in sinusoidal endothelial cells in control (a) and oleuropein (b) groups. In arsenic group (c), strong immunoreactivity in degenerating hepatocytes, weak immunoreactivity in sinusoidal endothelial cells and loss of immunoreactivity in the necrotic areas. Moderate cytoplasmic immunoreactivity in sinusoidal endothelial cells in arsenic plus oleuropein group (d).

lites of NO, were lower in arsenic given group via drinking water than that of the control. It has been shown that inorganic arsenate (As [V]) and arsenite (As [III]) suppresses the activity of eNOS. This may be one cause of the significant decrease of NO production in arsenic exposure [30]. Similarly, Pi et al. [30] detected significant decrease in plasma NO metabolites in humans received arsenic orally. They observed that the hepatic levels of BH_4 were considerably reduced in arsenic exposure. They suggested that arsenic exposure reduce eNOS activities by decreasing level of cofactor BH_4 [29]. These observations strongly suggest that arsenic exposure via drinking water may reduce NO productions.

Tissue iNOS activity has been shown to increase in variety of occasions as a result of oxidative stress [31,32]. iNOS is known to mediate catalytic formation of NO, and interactions of NO with some free radicals may end up with generation of products that interacts with cellular structures such as proteins and DNA [33]. In the current study, iNOS liver expression was also detected to increase in arsenic given mice. This result also parallels the degenerations observed in histomorphology indicating that the cellular degeneration and necrosis were the result of oxidative tissue stress.

Dietary oleuropein consumption varies greatly among the different geographical regions. Mediterranean basin is known to both produce and consume olive oil greatly. Although many beneficial effects of olive oil on health are known, there are only few studies on humans testing the daily consumption dose [34]. Oleuropein consumption even at high dosages was suggested to be nontoxic in human [35]. Due to its safe dosage limits 30 mg/kg/day was studied in the current investigation, and detected to show positive effects in reducing the oxidative stress and protein damage.

In conclusion, our current findings suggest that arsenic exposure via drinking water decreased NO production and cause protein damage. Dietary supplementation of oleuropein has been suggested to be beneficial in reducing the susceptibility to arsenic-induced lipid peroxidation

The results of the present study also confirms the previous results, and adds that oleuropein would be quite effective in ameliorating the histopathological changes as well as oxidative stress caused by arsenic in liver, kidney and brain tissues. The results indicate that oleuropein achieves these effects by improving antioxidant status and reducing protein damage.

Conflict of interest

The authors declare no conflict of interest.

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