EFFICIENCY OF FOLIC ACID AND ZINC CHLORIDE IN AMELIORATING ARSENIC-INDUCED TOXICITY IN SALIVARY GLAND AND TASTE BUDS OF ADULT MALE ALBINO RATS

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ABSTRACT

Background: Arsenic (As) in food and water can cause symptoms in soft tissues of mouth. Zinc metal and folic acid (a water-soluble B vitamin) act as antioxidants.

Objective: Assessment of the effects of arsenic on the submandibular salivary gland and taste buds in rats, and how folic acid and zinc chloride ameliorate these effects.

Methodology: Twenty-five rats were divided equally into 5 groups: (C); without any treatments, (HCl); got a daily oral dose of HCl for 15 days, (As); received 3 mg/kg b.w. orally of As III for 15 days. (As + Fa) and (As + Zn) received 0.1 mg/kg of folic acid and 2 mg/kg of Zncl2 respectively orally daily for 2 months after receiving As III. Plasma IL-6, IL-10, NO, and GSH measurements, Histological and immunohistochemical (GLUT-1 and cleaved caspase-3) examinations on submandibular salivary glands and taste buds were performed.

Results: Arsenic increased IL-6 and NO levels and decreased IL-10 and GSH levels significantly. Submandibular salivary gland and taste buds showed alterations as apoptosis and necrosis. Compared to control, salivary gland collagen fibers, GLUT-1, and taste buds cleaved caspase-3 increased significantly. Folic acid and zinc after arsenic exposure raised IL-10, GSH, and decreased IL-6 and NO significantly. Submandibular salivary glands and taste buds appeared nearly normal. Salivary gland collagen fibers, GLUT-1 immunoreaction, and taste buds cleaved caspase-3 immunoreaction decreased significantly versus arsenic group.

Conclusion: Oxidative stress from arsenic damages taste buds and submandibular salivary gland via apoptosis, necrosis, and inflammation. Folic acid and zinc chloride minimize that damage.

KEYWORDS: Arsenic, apoptosis, oxidative stress, folic acid, zinc chloride, healthcare.

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INTRODUCTION

Arsenic (As), a bright silver-gray element, is found naturally in many aspects of environmental media, including soil, water, air, and living things. Arsenic exposure in humans can occur when the Earth's crust is naturally disturbed by volcanic eruptions, earthquakes, or in industrial activity (Tchounwou et al., 2003; Habashi et al., 2013).

Arsenic is typically ingested by most people because of meals and water, which is the main way that people are exposed to it. Numerous health consequences are caused by prolonged exposure to arsenic in drinking water (WHO, 2017)

Arsenicosis, which is typically manifested by skin lesions with characteristics including hyperkeratosis, hypomelanosis, and hyperpigmentation, is a chronic health condition caused by prolonged absorption of arsenic over a safe amount (James et al., 2017). The respiratory, hepatobiliary, cardiovascular, central nervous, gastrointestinal, hematopoietic, and endocrine systems are examples of systemic symptoms that arsenic poisoning can have an impact on (Mazumder et al., 2000; Das et al., 2015).

Arsenic poisoning can also cause symptoms in the soft tissues of the mouth, such as the lips, gingiva, tongue, and buccal mucosa. Aphthous ulcer on the buccal mucosa, raindrop pigmentation of the tongue, patches of hypopigmentation of the tongue, hyperkeratotic nodules on the gingiva and the lips, and a crusted lesion of the lower lip are among the oral lesions (Syed et al., 2013). In a recent study, swollen vallate papillae were identified as a sign of arsenicosis (Sarwar et al., 2010). A significant side effect of arsenic exposure is buccal mucous membrane melanosis (Rahman et al., 2001).

Arsenic toxicity includes inhibition of DNA repair, oxidative stress, chromosomal aberrations, inflammation, apoptosis and cancer induction (Rao et al., 2017). In addition, arsenic induces cell hypoxia and altered stem cell phenotype populations (Dangleben et al., 2013; Srivastava & Flora, 2020).

Arsenic can mediate inflammatory changes as the relations between proinflammatory cytokines {interleukins IL-2, IL-8, IL-6, IL-12 and tumor necrosis factor alpha (TNF-α)} and anti-inflammatory cytokines (IL-4 and IL-10) regulate the immune response (Kany et al., 2019). Arsenic enhances pro-inflammatory reaction can lead to many diseases, as autoimmune disorders, cardiovascular events, infectious and allergic diseases (Ferrario et al., 2016; Rahman et al., 2021). Moreover, inflammatory genes polymorphisms may increase the risk of body function disorders and susceptibility to diseases in arsenic toxicity cases (Wu et al., 2013; Mu et al., 2019). So, the cytokines expression may be a possible and useful biomarker of arsenic tissues damage and toxicity.

Folate is considered as a water-soluble B vitamin. Metabolism of amino acid and nucleic acid is affected by folate, as folate acids play a role in production and maintenance of new cells by preventing DNA changes (Watanabe & Miyake, 2017). Also, folic acid has antioxidant activity via multiple mechanisms, including increase in total antioxidant capacity (TAC) and reduce ROS formation (Bahmani et al., 2014). As low folate levels are frequently seen in chronic inflammatory disorders, suggests that either chronic inflammation raises the need for folate, or that insufficient folate may be implicated in inflammation pathogenesis (Jones et al., 2019).

Zinc metal is a basic element for life as an essential element in structure and reaction site of many proteins and cellular function (Sharma et al., 2021). Zinc ion is liberated from its compound in oxidative stress situations, so zinc is considered as a pro-antioxidant (Maret, 2006). In addition, it regulates cytokine expression, suppresses inflammation, and activates antioxidant enzymes that go through reactive oxygen species, lowering oxidative stress (Olechnowicz et al., 2018).

Due to the toxicity of arsenic and the current environmental pollution caused by heavy metals, this study demonstrated the effect of arsenic metal biochemically on the blood, histopathologically (in submandibular salivary gland and taste buds), and immunohistochemically (GLUT-1 and cleaved caspase-3). Also, it assessed how folic acid and zinc chloride affected these tissues in adult rats that had been exposed to arsenic.

MATERIALS AND METHODS

1. Drugs and chemicals:

Arsenic III (As III) was bought from Sigma-Aldrich, ST. USA. It was dissolved into 0.12 N HCl (0.5 mg/kg body weight (b.w.) to obtain a stock solution of Arsenic trioxide. Zinc chloride ($Zncl_2$) was got from Piochem. Folic acid was purchased from Alpha Chemika Company.

ELISA was used (Dynatech Microplate Reader Model MR 5000, 478 Bay Street, Suite A213 Midland, ON, Canada) to detect plasma pro inflammatory marker interleukin-6 (IL-6) and anti-inflammatory marker interleukin-10 (IL-10) concentrations by ELISA kits from SinoGeneClon Biotech Co., Ltd, No.9 BoYuan Road,YuHang District 311112, Hang Zhou, China. Also, plasma oxidative markers as nitric oxide (NO) and antioxidant marker like reduced glutathione (GSH) levels were estimated using kits obtained from Biodiagnostic, Dokki, Giza, Egypt.

2. Experimental animals

Twenty-five male adult Wistar albino rats (200 - 250 gm) from Assiut University Joint Animal Breeding Unit, kept in 23 ± 2 ℃ temperature and 12 hours light / dark lighting cycle. All precautions for using and/or dealing with laboratory animals were taken into consideration and the Ethics Committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, according to the OIE standards for use of animals in research under the No. 06/2022/0002.

3. Experimental design

Twenty-five rats were divided into five groups; each group consisted of 5 rats:

Group 1 (C); served as a control without any treatments.

Group 2 (HCl); received a daily oral dose of 0.12 N HCl (0.5 mg/kg b.w.). Experimental

Group 3 (As); received a daily oral dose of 3 mg/kg b.w. ((Liu et al., 2021) of As III for 15 days.

Group 4 (As + Fa); received a daily oral dose of 3 mg/kg b.w. of As III for 15 days then received a daily oral dose of 0.1 mg/kg of folic acid (Ma et al., 2007) for 2 months.

Group 5 (As + Zn); received a daily oral dose of 3 mg/kg b.w. of As III for 15 days then received a daily oral dose of 2 mg/kg of $Zncl_2$ (Khater et al., 2017) for 2 months.

4. Collection and preparation of sample

After the end of experimental period, rats were anesthetized by ether inhalation, blood samples were taken in EDTA containing tubes from the jugular vein, then centrifuged for 10 minutes at 3000 rpm to obtain plasma that stored at -20 °C for estimation of IL-6, IL-10, NO and GSH.

Rats sacrificed, carefully dissected and specimens of submandibular salivary glands and tongues were processed for histological and immunohistochemical examinations.

5. Histological and histochemical examinations

Small pieces of the submandibular salivary gland and the circumvallate papillae of the tongue were immediately preserved in 10% neutral buffered formalin (pH 7.2) for histological and histochemical examinations. These selected sections were routinely processed using the paraffin-embedded technique. Then they were washed, dehydrated in ethanol solutions with increasing concentrations (from 70%

to 100%) to get rid of water, and cleaned in xylene before being embedded in wax. With a rotatory microtome, 5 μm thick sections of paraffin blocks were cut, and then the paraffin was removed by xylene. Lastly, the standard Hematoxylin and Eosin stains procedure were used for a general histological study, and the Masson trichrome stain was used to identify collagen (Bancroft & Gamble, 2008). Examination and photography were done with a digital camera (Toup Tek ToupView, Copyrightc 2019, Version:x86, Compatible: Windows XP/ Vista/7/8/10, China), ImageJ software, and a computer linked to a light microscope (Olympus CX31, Japan).

6. Immunohistochemistry of GLUT-1 and cleaved caspase-3

Formalin-fixed tissues of submandibular salivary glands and circumvallate papillae were put in 10% neutral buffered (pH 7.2). Sections of paraffin-embedded tissues were cleaned, rehydrated in ethanol solutions (100% to 70%), and then rinsed in water. Slides were boiled in 1 mM ethylenediaminetetraacetic acid (EDTA) for 10 minutes, and then sections were removed and treated with 3% hydrogen peroxide for 10 minutes to extract antigens. Each section was left in a blocking solution for an hour at room temperature. The primary GLUT-1 antibody (1:1000) (Novus Biologicals, LLC, USA) and cleaved caspase-3 antibody (1:1000) (Novus Biologicals, LLC, USA) were then added for 24 hours to salivary gland and circumvallate papillae sections respectively, followed by the secondary antibodies (1:5000) for 2 hours. The sections were stained with hematoxylin for 2-5 minutes after a 2–3-minute reaction with 3,3′-diaminobenzidine (Atia & Alghriany, 2021).

7. Statistical analysis

Data were represented as mean ± standard error of the mean (SEM). The results were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test using Prism software (version 8.0.1; GraphPad Software, Inc San Diego, CA, USA. Differences of p<0.05 were statistically significant.

RESULTS:

Biochemical parameters:

As shown in **table 1**, there was a significant decrease in IL-10 and GSH levels while there was a significant increase of IL-6 and NO levels in rats after arsenic administration compared to control and HCl groups (P < 0.001). However, there was no significant difference of all plasma parameters levels between control and HCl groups (P > 0.05).

Administration of folic acid and zinc after arsenic exposure significantly increased the levels of both IL-10 and GSH (P < 0.01) while they significantly decreased IL-6 and NO levels (P < 0.001) compared to arsenic group. There was no difference between zinc and folic acid treated groups in all measurements (P > 0.05).

TABLE (1) Shows the effect of folic acid and zinc chloride administration on plasma IL-6, IL-10, NO and GSH rats exposed to arsenic.

Treatment	C	HCl	As	As + Fa	As + Zn	P value
IL-6 (ng/L)	35.90 ± 2.529a	35.77 ± 2.797a	65.17 ± 4.565b	47.00 ± 2.506c	48.27 ± 1.940c	0.0001
IL-10 (Pg/L)	43.50 ± 3.451a	43.17 ± 1.909a	27.30 ± 0.981b	36.40 ± 1.100c	35.83 ± 2.491c	0.0001
NO (Mmol/L)	6.513 ± 0.520a	6.833 ± 0.322a	10.73 ± 0.306b	8.633 ± 0.322c	8.900 ± 0.265c	0.0001
GSH (mg/dl)	21.73 ± 0.566a	21.98 ± 0.475a	15.53 ± 0.877b	18.88 ± 0.733c	18.48 ± 0.65 c	0.0001

IL-6: Interleukin 6; IL-10: Interleukin 10; NO: Nitric oxide; GSH: Reduced glutathione.

Data are presented as Mean ± SE. Values in the same row followed by different superscript (a, b, c) are significant (P < 0.05).

2. Histopathological examinations:

Both the control and HCl groups were regarded as controls because there was no statistically significant difference between them in any of the histopathological or immunohistochemical findings.

Histopathological examination of salivary gland:

The Hematoxylin and Eosin-stained sections of submandibular salivary gland from control group revealed normal structure. Striated duct were lined with acidophilic columnar cells and normal serous and mucous acini **(Fig. 1a and b)**. Arsenic treated group **(Figs. 1c, d, e and f)** showed disorganization of submandibular salivary glands architecture with criteria of apoptosis as: peripheral chromatin condensation, nuclear fragmentation and apoptotic bodies with vacuolization of cells. Necrosis was seen in ductal and acinar cells. Ductal cells showed degenerating changes with vacuolization. Increasing of disorganized collagen fiber were detected. Moreover, cellular dysplasia as pleomorphism of nucleus and cells, hyperchromatism and abnormal mitosis (tripolar nucleus) were observed in some cells. Serous and mucous acini after treatment by folic acid showed normal orientation of ductal and acinar cells. Vacuolations are seen in some cells of intercalated and striated ducts **(Fig. 2a and b)**. Acinar cells after treated by zinc chloride showed normal orientation of cells with vacuolation of some ductal cells **(Fig. 2c and d)**.
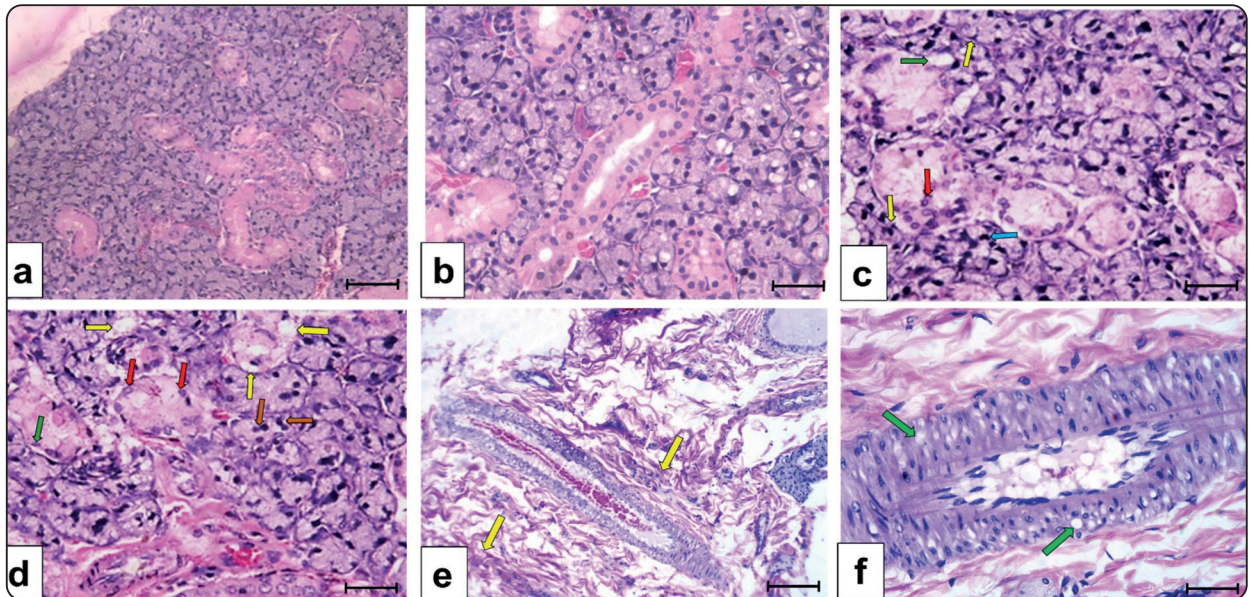

Fig. (1) Photomicrographs of sections of submandibular salivary glands stained by H&E, bars in a and e = 100 μm, in b, c, d and f = 50 μm. a and b: control group showing the normal architecture of ducts and acini. c: In arsenic group showing disorganization in submandibular salivary gland structure with criteria of dysplasia are seen as pleomorphism of nucleus (red arrow), kidney shaped cells with kidney nucleus (blue arrow), hyperchromatism and abnormal mitosis (tripolar nucleus) (yellow arrow), and vacuolization of cells (green arrow). d: arsenic group showing disorganization in salivary gland structure with necrotic ductal and acinar cells (yellow arrow) and criteria of apoptosis as peripheral chromatin condensation (red arrow), nuclear fragmentation (green arrow) and apoptotic bodies (brown arrows). Also, criteria of dysplasia are seen as pleomorphism of nucleus and cells and hyperchromatism e: In arsenic group showing an increasing of disorganized collagen fiber (yellow arrow). f: In arsenic group showing degenerating changes with vacuolization of ductal cells (green arrow).
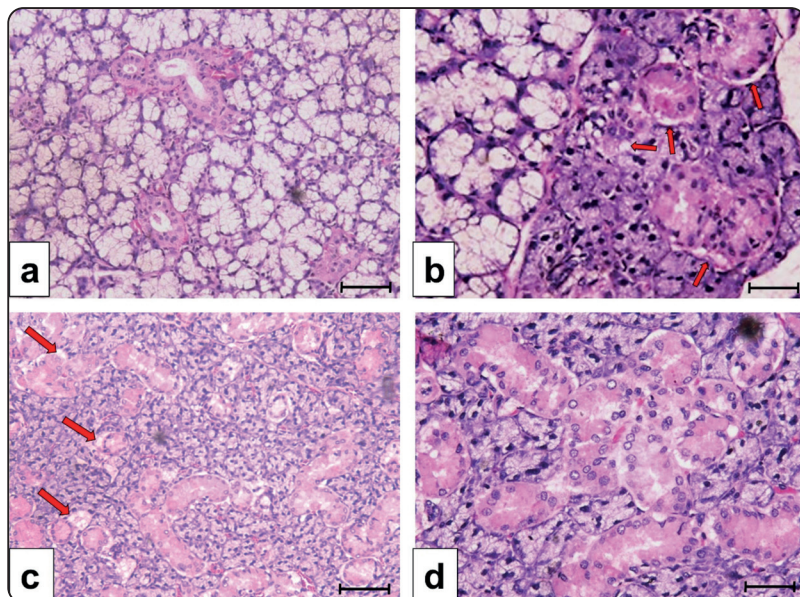
Fig. (2) Photomicrographs of sections of submandibular salivary glands stained by H&E, bars in a and c = 100 μm, in b and d = 50μm. a: mucous acini from folic acid treated group showing normal orientation of ductal and acinar cells b: serous and mucous acini from folic acid treated group showing normal orientation of cells. Vacuolization is seen in some cells of intercalated and striated ducts (red arrows). C and d: acinar cells after treated by zinc chloride showing normal orientation of cells with vacuolization of some ductal cells (red arrows).

Collagen fibers examination of salivary gland:

Masson's trichrome staining of control group sections indicated very low collagen fibers amount in stroma around blood vessels (**Fig. 3a**). Arsenic treated group showed great amount of collagen fibers in stroma around blood vessels as represented by the blue color (**Fig. 3b**). Minimum amount of collagen fibers in stroma were in folic acid treated group (**Fig. 3c**). In zinc treated group, moderate amount of collagen fibers in stroma were seen (**Fig. 3d**). Statistically, there was a significant increase in the percentage of collagen fibers in As treated group (P < 0.001) versus those of control group. But non-significant difference was in (As + Fa) and (As + Zn) groups when compared with control group (**Fig. 3e**).

Histopathological examination of taste buds:

The Hematoxylin and Eosin-stained sections of circumvallate papillae from the control group revealed the normal structure of the taste buds. The taste buds are onion-shaped intraepithelial organs. A small aperture called "the taste pore" was almost completely encircled by flat epithelial cells. The taste buds consisted of dark cells, light cells, and basal cells. The dark cells (supporting or type I cells) were slender in shape with elongated, darkly stained nuclei. The light cells (sensory or type II and III cells) were larger than the dark cells, with rounded vesicular nuclei and distributed between the dark cells. In the basal region of the taste buds, basal cells or type IV cells were observed (**Fig. 4a**). In Arsenic treated group (**Figs. 4b, c**), the circumvallate papillae had few taste buds and a small number of cells inside most of them. The buds lose their normal structure. Some taste buds had a small number of cells and wide spaces between them. The cells were hardly distinguishable and appeared degenerated with pyknotic nuclei. The folic acid-treated group showed a nearly normal appearance of taste buds with normal light and dark cells. Some cells appeared with pyknotic nuclei (**Fig. 4d**). In zinc treated group, some taste buds appeared normal with normal light and dark cells. Some taste buds appeared with few cells and empty spaces. Some cells had pyknotic nuclei (**Fig. 4e**).

3. Immunohistochemical examinations:

Immunohistochemistry of GLUT-1 in salivary gland

In control group, almost there was no immunoreactivity of GLUT-1 (**Fig. 5a**). Arsenic

treated group showed positive immunoreaction od GLUT-1in basolateral membrane of mucous acini, intercalated and striated duct. Also, immunopositivity of GLUT-1 in cuboidal cells of intercalated ducts and columnar cells of striated duct (Fig. 5b). Folic acid treated group showed an immune-negativity of GLUT-1 localized in nucleus of acinar cells but GLUT-1 immunoreactivity was detected in cells of the ducts (Fig. 5c). In (As + Zn) group, GLUT-1 immunoreactivity was seen in basolateral membrane of acini (Fig. 5d). Statistically, there was a significant increase in the GLUT-1 expression in As treated group ($P < 0.001$) versus those of control group. Significant decrease ($P < 0.001$) was in (As + Fa) and (As + Zn) groups when compared with As group. GLUT-1 expression increased significantly ($P < 0.001$) in the zinc-treated group compared to that in the folic acid-treated group. (Fig. 5e).

Immunohistochemistry of cleaved caspase-3 in taste buds

Immunohistochemical detection of cleaved caspase-3 protein of taste buds in control group showed a few immunoreactions (Fig. 6a). In As group, highly positive immunoreaction, as represented by brown color, especially in the nuclei of taste buds' cells was detected (Fig. 6b). This increase was statistically significant ($P < 0.05$) when compared with control group. Few immunoreactions were detected in most taste cells in the Folic acid-treated group (Fig. 6c). In (As + Zn) group, also few immunoreactions in most of the taste cells observed but few ones still with brown nuclei (Fig. 6d). Statistically, there was a significant decrease in the cleaved caspase-3 immunoreaction in (As + Fa) and (As + Zn) groups ($P < 0.05$) versus those of arsenic group. But non-significant difference was in (As + Fa) and (As + Zn) groups when compared with control group (Fig. 6e).
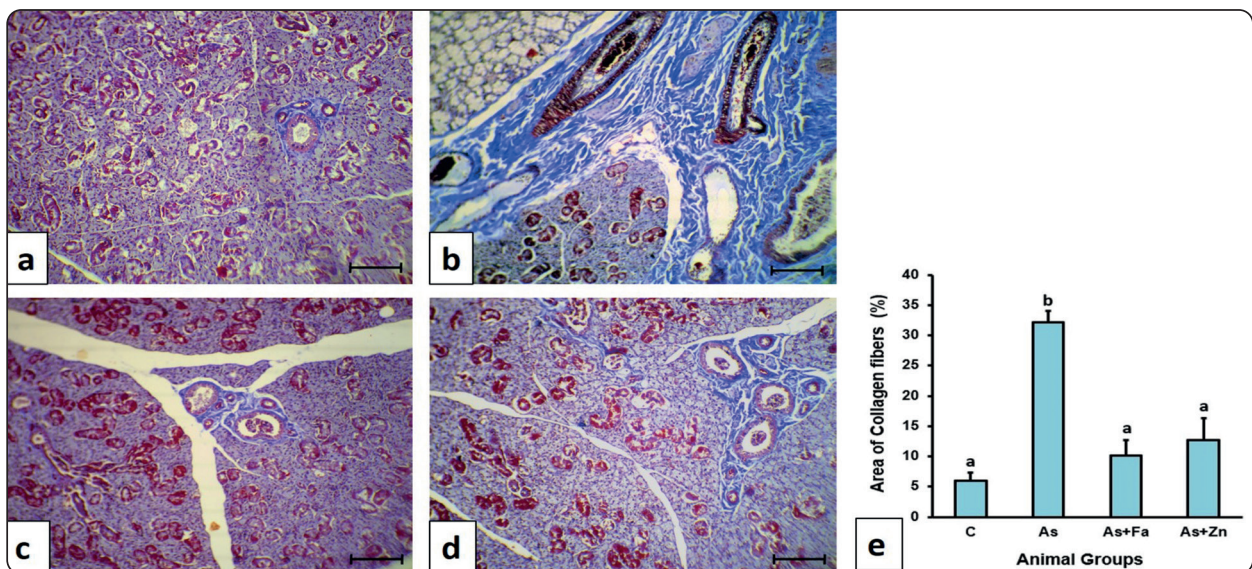

Fig. (3) Collagen fiber examination in different groups of animals. Photomicrographs of salivary gland sections stained by Masson's trichrome (a-d), bar = 50 μm. a: showing control group with very low collagen fibers, b: showing arsenic treated group with a marked increase in collagen fiber I as represented by the blue color. c: showing Folic acid treated group with the minimum collagen fibers, d: showing zinc chloride treated group with a noticeable amount of collagen fibers. e: Percentage of area of collagen fibers in the different experimental groups. Values in the column with unlike superscript letters are significantly different at ($P < 0.01$). Data represents mean ± S.E.M.
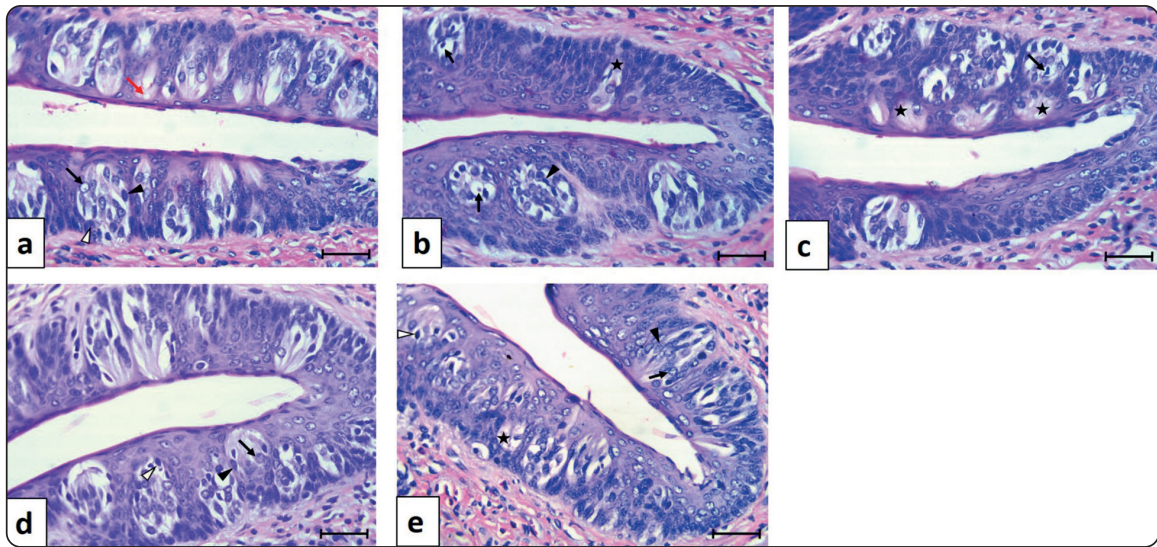
Fig. (4) Photomicrographs of taste buds in circumvallate papillae of rats from the experimental groups stained by H&E, bars = 50 μm. a: In control group showing onion shaped taste buds with apical pore (red arrow) and normal light sensory cells (↑), dark supporting cells (▲), and basal cells (Δ). b: In As group showing few taste buds and few number of cells inside most of them(asterisk). Degenerated cells (↑). Dark cells with irregular densely stained nuclei (▲). c: In As group showing taste buds lose their normal structure. Taste buds with few numbers of cells and wide spaces between them (asterisk). Degenerated cells with pyknotic nuclei (↑). d: In Fa group showing nearly normal appearance of taste buds with normal light (↑) and dark (▲) cells. Some cells appear with pyknotic nuclei (Δ). e: In Zn group, some taste buds are normal with normal light (↑) and dark (▲) cells. Some taste buds appear with few cells and with empty spaces (asterisk). Some cells are with pyknotic nuclei (Δ).
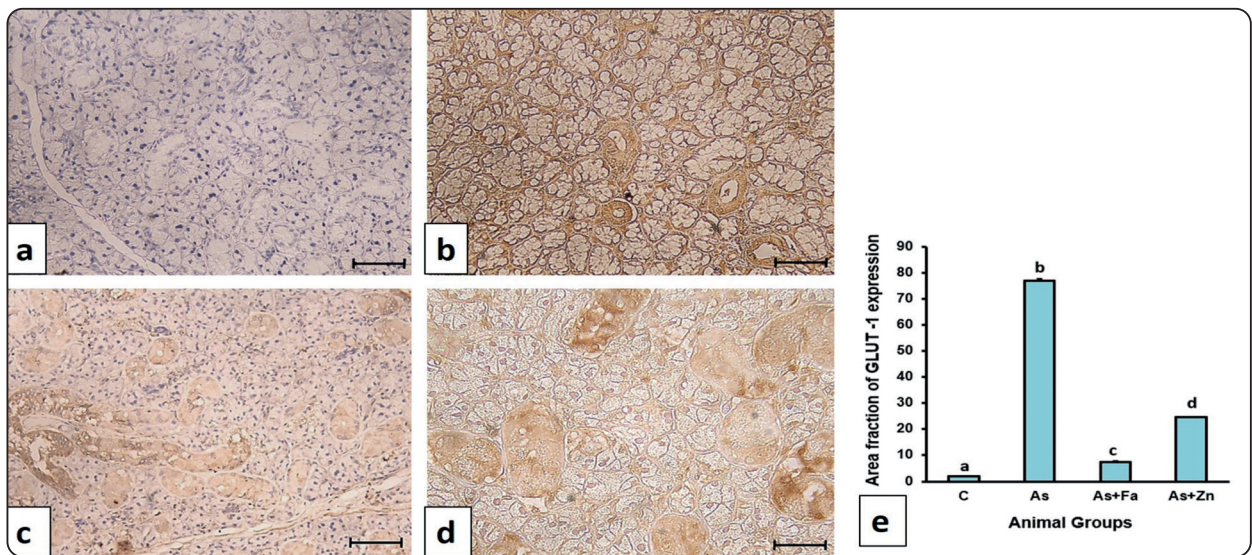

Fig. (5) Immunohistochemical detection GLUT-1 in salivary gland of rats from the experimental groups, bar = 100 μm. **a:** In control group showing no immunoreactivity of GLUT1 antibody **b:** In arsenic treated group showing positive immunoreaction in basolateral membrane of mucous acini, intercalated and straited duct. Also, immunopositivity of GLUT-1 in cuboidal cells of intercalated ducts and columnar cells of striated duct. **c:** In folic acid treated group showing GLUT-1 immunoreactivity in cells of the ducts. An immune-negativity of GLUT-1 is localized in nucleus of acinar cells. **d:** In zinc treated group showing immunohistochemical staining of GLUT-1 in basolateral membrane of acini. Also, strong immune-positivity reaction of GLUT-1 in cells of the duct. An immune-negativity of GLUT-1 is localized in nucleus of acinar cells. **e:** Area fraction of GLUT-1expression in the different experimental groups. Values in the column with unlike superscript letters are significantly different at (P < 0.01). Data represents mean ± S.E.M.
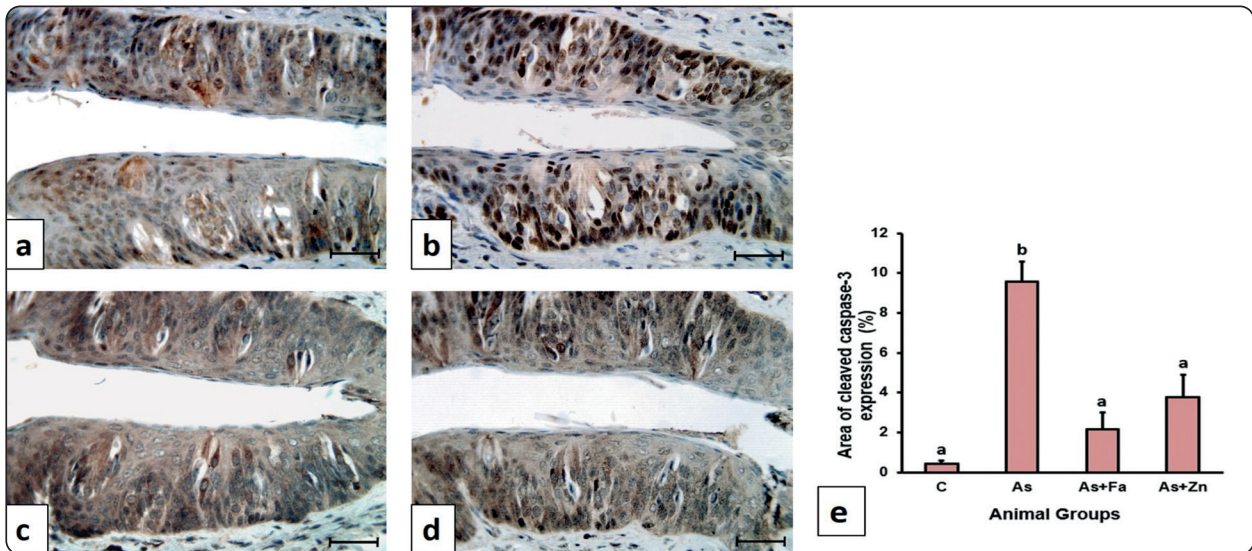
Fig. (6) Immunohistochemical detection of cleaved caspase-3 protein of taste buds in circumvallate papillae of rats from the experimental groups, bar = 50 μm. (a): In control group, showing few immunoreactions for cleaved caspase-3. (b): In As group, showing highly positive immunoreaction as represented by brown color specially in the nuclei of taste buds' cells. (c): In Fa group, showing few immunoreactions in most of the taste cells. (d): In Zn group, showing few immunoreactions in most of the taste cells but few ones still with brown nuclei. (e): Percentage of area of cleaved caspase-3 protein expression in the different experimental groups. Values in the column with unlike superscript letters are significantly different at (P < 0.01). Data represents mean ± S.E.M.

DISCUSSION

The effects of chronic arsenic exposure, particularly when it occurs through drinking contaminated ground water, are detrimental to human health. It influences prenatal development, cancer, and dermatological conditions (Chakraborti et al., 2013).

Numerous studies have been conducted to determine how arsenic affects people's overall health. There is, however, a dearth of knowledge on intraoral findings and exposure to arsenic so we aimed in this study to focus on the effect of arsenic metal biochemically on the blood, histopathologically (in submandibular salivary gland and taste buds), and immunohistochemically (GLUT-1 and cleaved caspase-3). Also, it assessed how folic acid and zinc chloride affected these tissues in adult rats that had been exposed to arsenic.

Biochemically, our results showed increased proinflammatory interleukin IL-6 while there was

a decreased anti-inflammatory IL-10 in arsenic group compared to control and HCl groups. That agrees with (Zhang et al., 2022) who explained arsenic immunotoxicity to cell-mediated response and altered inflammatory cytokines as he found increased IL-6, IL-12 and IL-8 levels in arsenic-exposed cases in comparison to the control one.

Also, (Biswas et al., 2008) recorded that arsenic exposed individual showed significant decrease in lymphoproliferation and Th1/Th2 production of IL-10, IL-4, IL-2, IL-5, TNF-α and IFN-γ compared with unexposed individuals. Besides, (Wu et al., 2003; Salgado-Bustamante et al., 2010) added that among genes, prolonged exposure to arsenic may induce current inflammation that could contribute to alternation in inflammatory genes, some up-regulated, including IL1B, IL6, CD14 and CCL_2 while others down-regulated, including IL11, TNF, IL10, CXCL2, and CCR1.

Moreover, this research approved that arsenic increased nitric oxide NO while there was decreased

glutathione GSH levels in arsenic group compared to the control and HCl groups. That is agree with Ganger (Ganger et al., 2016)who found that arsenic exposure generates reactive oxygen species (ROS) including superoxide anion (O_2 –), nitric oxide (NO –), hydroxyl radical (• OH), singlet oxygen (1 O_2), hydrogen peroxide (H_2O_2) which led to tissue damage.

In addition, (Flora et al., 2007; Tripathi et al., 2022) suggested that lipid peroxidation and ROS generation in case of As exposure led to laboratory animals genotoxicity and brain damage due to oxidative stress, along with nitrosative stress. As nitric oxide (NO) and H_2O_2 enhance each other production (Zhao, 2007) so, superoxide can rapidly interact with NO, causing production of peroxynitrite that breaks down DNA, protein, and lipid during oxidative stress (Matsubara et al., 2015). Also, (Amal et al., 2020) reported that As led to a Ca^{2+} release in cortical neurons and activation of NO-mediated apoptosis in cultured primary neurons.

Besides, (Maiti & Chatterjee, 2001; Shen et al., 2013) said that arsenic interacts with sulfhydryl containing biomolecules as pyruvate dehydrogenase (PDH), reduced glutathione (GSH), glutathione peroxidase (GPx) and glutathione reductase (GR), decreasing their activity. So, arsenic exposure leads to oxidative stress that includes either direct elevation of free radical generation or inactivation of antioxidant enzymes which are responsible for counteracting and scavenging free radicals (Ganger et al., 2016).

Glucose transport protein 1 (GLUT-1) controls the transport the glucose to the cells which is responsible for maintenance of homeostasis of biological reactions. In physiological situations, GLUT-1 levels are increased with increasing in metabolic need. While in pathological situations, an increasing in levels of GLUT-1 proteins is an indicator for tumor aggressiveness and progression as in case of neoplasms (Mori et al., 2007; Thorens & Mueckler, 2010).

In submandibular salivary gland, our histopathological data revealed by H&E staining that the control salivary gland tissues of sub mandibular showed normal orientation of acinar cells and ducts. Regarding the arsenic group, the photomicrograph pictures showed criteria of epithelial dysplasia, apoptosis and necrosis of acinar cells and ducts.

This is supported by (Chattopadhyay et al., 2002) who reported that arsenic induce cell death by necrosis and apoptosis in brain tissue cultures with an incensing in oxidative stress and membrane damage. Also, (Polat et al., 2018) stated that arsenic induces oxidative stress and apoptosis.

The immunohistochemical staining in normal submandibular tissues showed a negative immunoreactivity of GLUT-1 this agrees with (Cetik et al., 2014) who stated that there was no immunoreactivity of GLUT-1 in control rodent submandibular glands. While the arsenic group showed the strongest positivity of GLUT-1. This may be due to the ability of arsenic to change the expression of certain proteins that compose saliva (Guber et al., 2021)**.** Moreover, it may be due to arsenic induced hypoxia (Srivastava & Flora, 2020).

In the circumvallate papillae, this study showed that the H&E-stained sections of circumvallate papillae from the control group revealed the normal structure of taste buds. On the contrary, in the Arsenic-treated group, few taste buds appeared, and most of them had a small number of cells and wide spaces between them. The cells were hardly distinguishable and appeared degenerated with pyknotic nuclei. (Pai et al., 2007) investigated the possibility that missing taste buds are replaced by lingual epithelium because of epithelial proliferation to fill the space left by taste buds. (Shankar et al., 2023) stated that the As-toxicity has caused a variety of negative effects as organ failure and oxidative stress. Oxidative stress caused by long-term

exposure to arsenic is linked to methyl deficiency, and the loss of DNA methylation in animals may be the cause of the histological changes (Mohammod, 2015). Free radicals produce lipid peroxidation, which in turn causes oxidative deterioration of the bilayer matrix of cell membrane lipid, which in turn causes deactivation of membrane-bound receptors and increased tissue permeability which result in cell malfunction and eventual death (Kumar et al., 2020).

Caspase-3 immunohistochemical detection has previously been established to be a reliable approach to detect apoptosis even before any morphological cellular changes associated with apoptosis take place (Gown & Willingham, 2002; Hassan et al., 2019). Caspase-3 is one of the most important executional caspases, which are responsible for cleaving various intracellular compartments in the context of apoptosis (Kopeina et al., 2018). The nuclear transfer of activated caspase-3 is thought to be a critical step in the beginning of apoptosis (Köhler et al., 1999).

In the current study, immunohistochemical detection of cleaved caspase-3 protein of taste buds in control group showed a few immunoreactions. Arsenic administration increased the immunoreactivity of caspase-3 significantly versus those in control group. Folic acid and zinc administration significantly decreased the cleaved caspase-3 immunoreaction when compared with arsenic group. Non-significant difference was between folic acid and Zinc groups when compared with control group.

This is in line with the suggestion made by (Zeng & Oakley, 1999), who believed that apoptosis is the major mechanism for taste bud cell death. In an earlier study by (Morales et al., 2008), it was shown that As2O3 raises the levels of BH3-only proapoptotic (Bcl-2 homology region 3 (BH3)-only proapoptotic) proteins in myeloma and lowers the levels of antiapoptotic proteins. additionally, the extrinsic apoptotic pathway, which involves

Fas/FasL, also participates in arsenic-induced keratinocyte apoptosis (Liao et al., 2004; Mu et al., 2019).

In folic acid and zinc treated groups, our biochemical results proved that zinc and folic acid succeeded in decreasing IL-6 and increasing IL-10 plasma levels than in arsenic group. That agrees with (Cianciulli et al., 2016)who found that folic acid treatment inhibited TNF-α and IL-1β, and iNOS dependent NO production as well as increased appearance of the anti-inflammatory cytokine IL-10 in LPS-activated BV-2 cells. (Bao et al., 2010) showed that zinc decreased TNF-α, and Il-6 plasma concentrations suggesting that it may have a protective effect in atherosclerosis because of its anti-inflammatory and antioxidant functions.

Additionally, zinc and folic acid worked to decrease NO and increase GSH plasma levels than that in the arsenic group. Our result agrees with (Ruttkay-Nedecky et al., 2013) who showed that zinc antioxidant characters have been referred to GSH synthesis, metallothionein (MT) induction with regulation of both oxidant production and redox signaling. MT acts as an antioxidant and helps in ROS scavenging in stress conditions. In addition, (Ferretti et al., 2007) said that Zn may compete with As for binding to GSH sulfhydryl groups because Zn has high ability to interact with it, thus protecting it from oxidation.

The improvement of antioxidant state following addition of folic acid was illuminated either by its directly antioxidative result, or by the reduction of homocysteine concentration. Its supplementation directly brings down oxidative stress markers and strengthen serum total antioxidant capacity and glutathione concentrations. Besides, it is a co-substrate in homocysteine re-methylation which is converted to methionine as increased homocysteine mediates endothelial cell toxicity by increasing H_2O_2 production and affecting antioxidant defense systems (Froese et al., 2019; Asbaghi et al., 2021).

Moreover, supplementation of folic acid improves arsenic methylation that reduces the arsenic toxicity risk. Folic acid that used in dietary supplement, is transformed to folate inside the body; arsenic is methylated by folate-dependent one-carbon metabolism to dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA) (Gamble et al., 2006). Also, (Gamble et al., 2007) said that folic acid addition may decline blood arsenic concentration and make it simpler to get rid of arsenic in the urine.

Histopathologically, in folic acid and zinc chloride treated groups after arsenic application, the submandibular salivary gland tissues by H&E stain showed normal orientation of ductal and acinar cells. Vacuolations are seen in some cells of intercalated and striated ducts. This may be explained by a study of (Al-Basrawi & Al-Mashhadane, 2020) which revealed that folic acid enhances total antioxidant properties of saliva. And by (Kostecka-Sochoń et al., 2018) who reported that the zinc has an antagonist feature against metal toxicity as the zinc has antioxidant properties.

Compared to the arsenic application group, the strength of immunohistochemical staining of GLUT-1 in submandibular tissue is less in folic acid treated group and zinc chloride treated group. Concerning the folic acid treated group, this may be due to the ability of folate to create methionine, which is main resource of methyl compound in methylation steps through removing of arsenic toxicity (Rao et al., 2017).

In zinc chloride treated group, these results may be explained by a study of (Kumar et al., 2010) who formed that the property of zinc to decrease oxidative stress by a decreasing in levels of ROS with an increasing in antioxidant capacity in arsenic-treated rats by zinc.

Folic acid and Zinc administration to arsenic treated rats in our study returned the taste buds to a nearly normal appearance. This improvement was brought about by folic acid rather than zinc. (Mohamed & Nor-Eldin, 2018) explained that folic acid is used as a supplement to combat the effects of deterioration on tissues because it is crucial for the synthesis of DNA and RNA, which promotes quick cell division and growth. (Mansour & Mossa, 2010) explored that the protective effect of zinc may be the consequence of zinc's direct involvement in the scavenging of free radicals, thereby enhancing the antioxidative capacity of cells.

Folic acid and zinc administration to arsenic treated rats in the current study significantly decreased the cleaved caspase-3 immunoreaction when compared with arsenic group. Non-significant difference was between folic acid and Zinc groups when compared with control group.

These findings are consistent with those of (Koohpeyma et al., 2020) who found that folic acid significantly decreased the levels of caspase-3 in the cerebellum and suggested that folic acid, in addition to having antioxidant properties, can protect the cerebellum against homocysteine-mediated neurotoxicity by regulating the expression of proteins involved in apoptosis regulation in the rat cerebellum.

Zinc chloride ($ZnCl_2$) has been shown in numerous studies to prevent apoptosis in vitro (Salesa et al., 2021). Zinc has been discovered to inhibit caspase-3, despite the fact that it was once believed to stop endonuclease activity in its late phase. A class of proteolytic enzymes known as caspases is essential for a cell to commit to the apoptotic process. This series of events culminates in caspase-3, which causes poly (ADP-ribose) polymerase to be proteolyzed. This activity is what comes before endonuclease activity, which is a key component of DNA repair mechanisms. $ZnCl_2$ might make it possible for more cells to survive apoptosis by interrupting the cascade at a point before morphological alterations (Kown et al., 2000).

CONCLUSION

Arsenic metal is toxic agent to submandibular gland and taste buds' tissue, it induces inflammation, apoptosis, and necrosis. Folic acid and zinc chloride treatments decrease toxic effects of arsenic on submandibular and taste buds' tissue. According to histopathological study, folic acid showed a better repairing effect than zinc supplementation against arsenic toxicity.

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