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Research Article

Pathological and Immunohistochemical Studies on the Ameliorating Effect of *Spirulina Platensis* against Arsenic Induced Reproductive Toxicity in Female Albino Rats

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ABSTRACT

The aim of this study was to determine the effect of sodium arsenate (Na_3AsO_4) on the female reproductive organs, and to study the effect of *Spirulina platensis* (Sp) as an ameliorating agent in the arsenic-induced toxicity. 40 female Wistar albino rats were used. The rats were divided into four equal groups, 10 rats in each group; control group and three groups that received spirulina (Sp), sodium arsenate, and sodium arsenate plus spirulina respectively, for 2 months. Results showed that the body weight in female rats was significantly reduced in arsenic-treated group compared to the control, while the co-treatment with Sp significantly restored the body weight. Arsenic significantly increased serum malondialdehyde (MDA) and significantly reduced serum glutathione (GSH) activities in comparison with the control group. Spirulina co-treatment significantly restored GSH levels and significantly reduced MDA in comparison to arsenic-treated group. Histopathologically, uterus and ovaries of arsenic-treated group showed different degenerative changes which improved with the Spirulina co-treatment. Immunohistochemistry determined the role of nuclear erythroid 2-related factor 2 (Nrf2) in the arsenic toxicity and *Spirulina Platensis* mechanism of protection in arsenic-induced toxicity. In conclusion, arsenic-induced toxicity in female rats could be ameliorated by *Spirulina platensis* co-administration through Nrf2 pathway.

Key words: Sodium arsenate, *Spirulina platensis*, Uterus, Ovaries, Histopathology, Nrf2

INTRODUCTION

Arsenic is one of the most important worldwide environmental toxicants, which is found in air, water and soil. Arsenic is mainly distributed in the environment through using pesticides and herbicide, burning coal and treated wood, mining wastes, smelting metal and glass. The main exposure to arsenic is occupational and the most frequent reason of poisoning is the use of contaminated water (Goudarzia *et al*., 2018). Arsenic (AS) is widely distributed in the environment due to its natural and anthropogenic sources. High levels of inorganic arsenic are found in ground water in many regions of the world as a result of geochemical processes posing serious chronic health risks to humans (Rajesh *et al*, 2010). Reports since early 19th century have confirmed a relationship between arsenic exposure and morbidities. Drinking water is the most common source of arsenic exposure (Firdausa *et al*., 2018). Chronic arsenic exposure may affect a number of organs. Arsenic exposure has been associated with health problems including developmental abnormalities, diabetes, hematological disorders, neurological and reproductive problems and cancer (Rajesh *et al*, 2010). Arsenic may cause obvious damage in various organs, including female reproductive system, as manifested by disruption of the circulating levels of gonadotropins and estradiol, leading to degeneration of luminal epithelial, stromal and myometrial cells of the uterus and abrogation of the estrogen-signaling pathway in female (Chatterjee and Chatterji, 2010). Arsenic toxicity expedites generation of reactive oxygen species (ROS). Mitochondrial dysfunction contributes to enhanced intracellular reactive oxygen species (ROS) levels, which further elicit damage to the cells and mitochondria itself (Firdausa *et al*., 2018). Arsenic exposure leads to the increased production of reactive oxygen species (ROS) and other toxic intermediates via biotransformation of arsenic, which may subsequently cause oxidative stress and alterations of cellular system. Glutathione peroxidase (GSH-Px) is regarded as the first line to protect the membrane lipids

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from oxidative damage. When this enzymatic scavenger cannot counteract excessive ROS, lipid peroxidation will occur. One of the lipid peroxidation metabolites is malondialdehyde (MDA), which arises primarily from peroxidative cleavage of polyunsaturated fatty acids in biological systems and is also used as an biomarker to balance oxidative damage (Zhao *et al*., 2017). Chelation therapy using synthetic chelating agents is the only available therapeutic method for arsenicosis. However, related adverse side-effects such as chelation of essential metals and arsenic redistribution in tissues mostly limited their clinical use. Also, dietary antioxidants are known for a long time for their effectiveness against oxidative stress-related complications. The correlation between arsenic toxicity and oxidative stress provides an indisputable platform for phytochemicals which may serve as a useful preventive/therapeutic approach as recently recommended by World Health Organization (WHO) (Firdausa *et al*., 2018). Microalgae have been known as food and animal feed; they grow in freshwater and marine (Vigani *et al*., 2015). Spirulina is a photosynthetic cyanobacterium that is used commercially as a dietary supplement and food additive. The extensive production of Spirulina is due to its original chemical composition [proteins, polyunsaturated fatty acids, and vitamins]. Besides, it is a source of bioactive components like, phycocyanin, β-carotene, and allophycocyanin, which has anti-inflammatory and antioxidant properties (Wang *et al*., 2007). The studies conducted in the past using *Spirulina platensis* as a supplement have proved that it has an ability to counteract the toxicity caused by many medications and chemicals (Banji *et al*., 2013).

Aim of work: This study was aimed to investigate the effect of arsenic toxicity on the female reproductive system in albino rats and evaluating the ameliorative potential of *Spirulina platensis* against the arsenic-induced toxicity by its co-administration with arsenic.

MATERIALS AND METHODS

Chemicals

Pure *Spirulina Platensis* powder was obtained from Arab Academy for Science, Technology and Maritime Transportation, Alexandria, Egypt. Sodium arsenate (Na_3AsO_4) obtained from the representative of Fisons Scientific Apparatus Ltd. UK in Egypt. All kits for biochemical analysis were purchased from Biodiagnostics, Egypt. All other chemicals used in the experiment were of analytical grade.

Animals

40 female Wistar albino rats weighing 125 ± 20 gm were randomly divided into four equal groups: Control and three treatment groups (As, Sp and As+Sp) (10 rats in each group). Rats were obtained from Helwan Animal Colony belonging to VACSERA. The animals were housed in standard cages, kept in a ventilated room under controlled laboratory conditions of normal light-dark cycle (12 hours light/dark) and temperature (25 ± 2˚C). Standard laboratory chow and water were provided ad libitum. Animals allowed to acclimatizing for two weeks before starting the study. This experimental protocol was approved by the institutional Animal care and Use Committee (CU-IACUC), Cairo university, Egypt (approval No. CU-II-F-37-18).

Experimental design

Female rats were divided randomly into four equal groups. The first group served as a control group that received standard laboratory chow and water ad libitum. The second group received Sp (300 mg/ kg bwt) dissolved in water by oral route daily. The third group daily received orally sodium arsenate (5 mg/kg bwt) dissolved in normal saline. Fourth group was given Sp (300 mg/kg body weight) and sodium arsenate (5 mg/kg body weight). The treatment was continued for two months. Rats were euthanized by decapitation at the end of the experimental period.

Body weights

The final body weight of rats was recorded by weighing each rat in all groups for detection of body weight changes.

Lipid peroxidation and reduced glutathione

After euthanizing the rats, blood samples were collected from the retro-orbital venous plexus in a sterile centrifuge tubes just before necropsy. Blood samples were left to clot at room temperature and centrifuged at 3000 rpm for 15 minutes and the sera were separated and stored at -20˚C as aliquots for further biochemical analysis. Lipid peroxidation was evaluated by measurement of serum malondialdehyde (MDA) (Ohkawa *et al*., 1979) based on the formation of thiobarbituric acid reactive substances (TBARs) and expressed as the extent of malondialdehyde (MDA) production. The non-enzymatic antioxidant biomarker, reduced glutathione (GSH) was assessed (Beutler *et al*., 1963).

Postmortem and histopathological examination

After euthanizing, rats were subjected to careful postmortem examination. Specimens from uterus and ovaries were collected and fixed in formal saline 10% then washed, dehydrated, cleared and embedded in paraffin. The paraffin embedded blocks were sectioned at 4-5 micron thickness and stained with Hematoxylin and Eosin (Bancroft *et al*., 2012).

Histomorphometric studies of uterine and ovarian tissues

The size of endometrial glands and thickness of endometrium and myometrium were measured by light microscope (Olympus BX50, Japan). The number of different follicles from three sections of each ovary per / rat (n=10) were counted microscopically using TSView version 6.2.4.5 software.

Immunohistochemistry

Formalin-fixed, paraffin-embedded 4 µm sections were fixed into poly-L-lysine coated slides (Thermo Scientific, Karlsruhe, Germany). After deparaffinization and rehydration, the slides were immersed in buffer Target Retrieval Solution, pH 9.0 (Dako, Glostrup, Denmark). Peroxidase Blocking Solution (Dako, Glostrup, Denmark) was used to block activity of

endogenous peroxidase. The slides were incubated with 1 mg/ml of the nuclear erythroid 2–related factor 2 (Nrf2) Rabbit Anti- Human Polyclonal Antibody (LS-C118543 – LSBio; LifeSpan Biosciences, Seattle, WA, USA) for 30 min at room temperature and immunostained with DAKO Real™ Envision™ Detection System Peroxidase/DAB+, HRP Rabbit/Mouse (Dako, Glostrup, Denmark). Hematoxylin was used as a counterstain.

Statistical analysis

All results were expressed as mean ± SD (Standard Deviation). Statistical analyses were performed using the SPSS version 24.0 statistical analysis package (SPSS, Inc., Chicago, IL, USA). The parametric test one-way ANOVA was used for data analysis and comparison was done using Tukey post-hoc test. In all calculations, a difference at P<0.01 or P<0.001 was considered as significant.

RESULTS

Body weights

Arsenic treated group showed significant reduction in the rats body weights (P<0.01 and P<0.001) in comparison with the control group. Co-administration of *Spirulina* significantly recovered the body weight in comparison with As-treated group as illustrated in (Fig. 1).

Antioxidant markers

Serum levels of GSH and MDA in different experimental groups was shown in (Fig. 2). The results revealed a significant depletion (P<0.01 and P<0.001) of serum GSH in the As-treated group in comparison with control one. While the co-administration with Spirulina platensis resulted in a significant increase (P<0.01 and P<0.001) in serum antioxidant capacity (GSH) in comparison with As-treated rats. On the other hand, serum MDA in As-treated rats showed a significant elevation (P<0.01 and P<0.001) in comparison with the control group. Meanwhile, the co-treatment with Spirulina platensis revealed a significant decrease of MDA levels in comparison with As-treated group (P<0.01 and P<0.001).

Gross findings

No any abnormal gross finding was observed during the postmortem examination.

Histopathological findings

Microscopically, uterus of control rats as well as spirulina treated group showed the normal histological organization and architecture of uterus with normal invagination of uterine lumen and normal uterine glands (fig. 3a). On contrary, uterus of the arsenic treated rats showed degeneration of the uterine endometrial lining epithelium with a decrease in the invaginations of the uterine luminal epithelium than control one, endometrial glands also affected as their number was clearly decreased in the As-treated group in compare to the control group (fig. 3b), some of them appeared atrophied or not well differentiated. All these remarkable changes appeared in the uterus were less noticed and clearly abated in group that co-administered with spirulina (fig. 3c) as the number of endometrial glands and endometrial luminal invagination were nearly returned to normal structure.

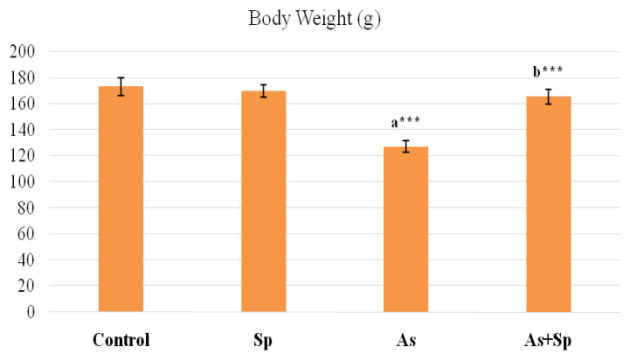

Fig. 1: Female average body weight (g) among different experimental groups. Sp: *Spirulina*; As: arsenic; As+Sp: Arsenic and *Spirulina*. The values are expressed as the means ± SD, where n=10. Superscript a refers to a significance from control group, Superscript b refers to a significance from As group. *** refers to P<0.001.

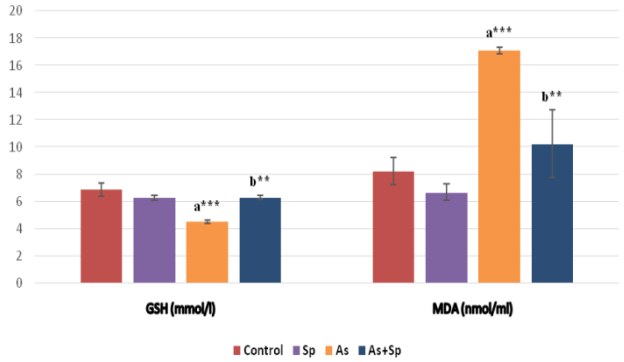

Fig. 2: Serum GSH (mmol/l) and serum MDA (nmol/ml) among different experimental groups. Sp: *Spirulina*; As: arsenic; As+Sp: Arsenic and *Spirulina*; GSH: Reduced glutathione; MDA: Malonaldehyde. The values are expressed as the means ± SD, where n=10. Superscript a refers to significant from control group, Superscript b refers to significant from As group. ** refers to P<0.01, *** refers to P<0.001.

Table 1: Effect of sodium arsenate and spirulina co-administration on size of endometrial glands, thickness of endometrium and myometrium in female albino rats

Group	Size of endometrial glands (µm)	Endometrial thickness (µm)	Myometrial thickness (µm)
Control	33.29±3.4 [a]	64.24±4.3 [a]	66.73±3.1 [a]
SP	33.1±4.1[a]	62.11±2.3[a]	66. 56±2.4[a]
AS	22.38±4.7 [b]	50.64±7.2 [b]	42.50±3.4 [b]
AS+SP	30.79±4.3 [a]	59.21±4.5 [a]	61.34±2.8 [a]

Sp: *Spirulina*; As: arsenic; As+Sp: Arsenic and *Spirulina*. The values are expressed as the means ± SD, where n=10. Superscript refers to significance from control group. *P*< 0.01 was considered significant.

Table 2: Effect of sodium arsenate and spirulina co-administration on the number of ovarian follicles

Group	Number of healthy ovarian follicles
Control	20.14±1.12[a]
SP	18.21±2.1[a]
AS	6.11±0.54[b]
AS+SP	11.15±1.20[c]

Sp: *Spirulina*; As: arsenic; As+Sp: Arsenic and *Spirulina*. The values are expressed as the means ± SD, where n=10. Superscript refers to significance from control group. *P*< 0.01 was considered significant.

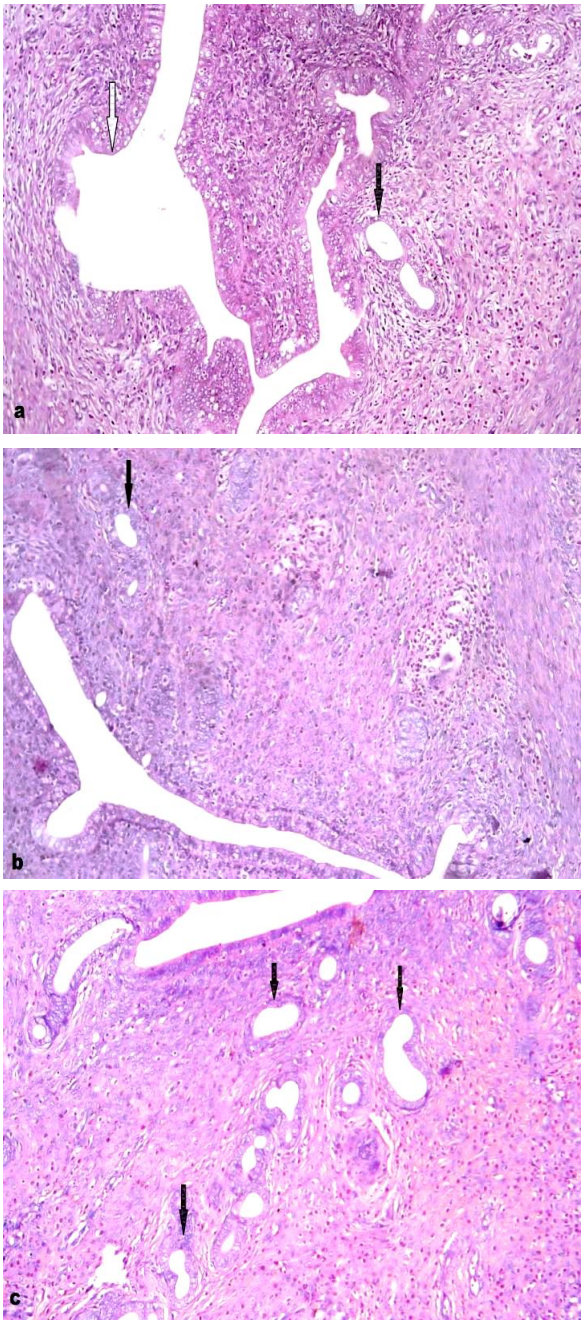

Fig. 3: Micrograph, uterus, female rat. Control untreated group showing the normal histological structure of the uterus with normal invagination of uterine lumen (white arrow) and normal uterine glands (black arrow) **(a)**. In arsenic treated group the uterus appeared with decreased size and invaginations of the uterine lumen with decreased endometrial glands number (arrow) **(b).** Recovery was noted in sections of uterus co-administered with spirulina as the number of uterine glands showed noticeable increase (arrows) **(c). (H & E X100).**

Concerning ovaries, examined sections from control rats as well as spirulina treated rats revealed the normal histological structure with normal number of healthy ovarian follicles and less number of atretic follicles ((fig. 4a). Ovaries of As-treated rats showed a great reduction in the number of ovarian follicles with increased number of atretic follicles in comparison with control group (fig. 4b). Co-treatment with spirulina showed a notable improvement in all previous change as the function of ovaries returned nearly normal with increased the number of healthy ovarian follicles (Fig. 4c).

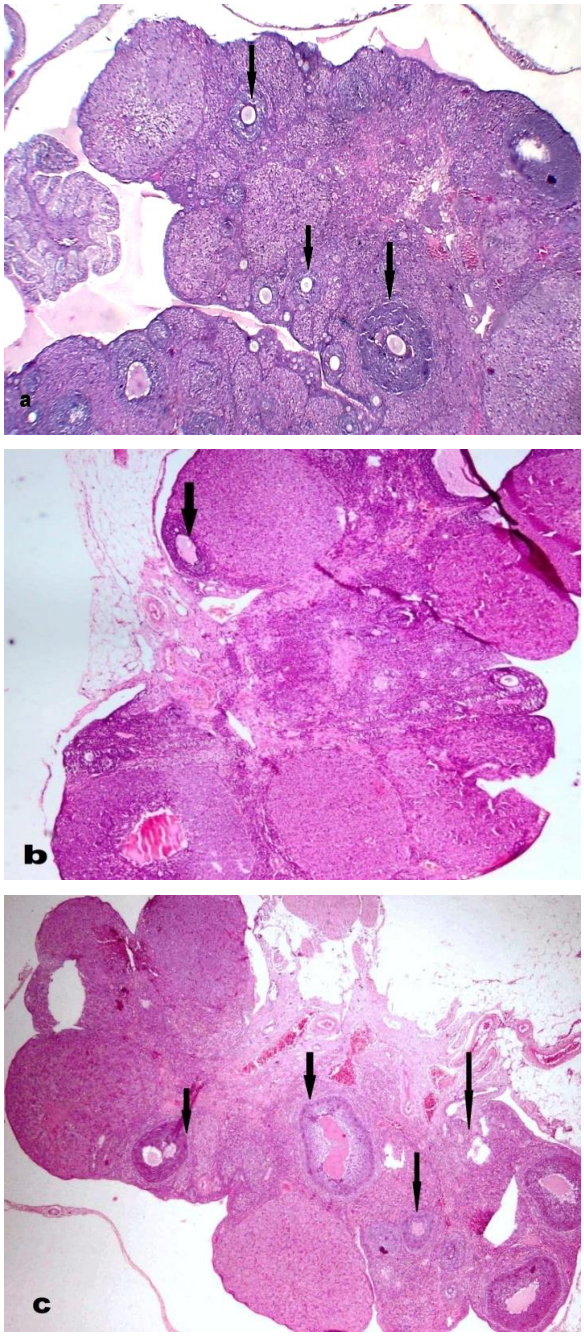

Fig. 4: Micrograph, ovary, female rat. Control untreated group showing considerable number of ovarian healthy follicles (arrows) **(a)**. In arsenic treated group the ovary showing dramatic decrease in the ovarian follicles (arrow) **(b)**. In groups co-administered with spirulina, the ovary restored its function with increased healthy ovarian follicles number (arrows) **(c)**. **(H & E X40).**

Histomorphometry of uterine and ovarian tissues

Concerning uterus, the size of endometrial glands and thickness of endometrium and myometrium were significantly reduced ($P<0.01$) in arsenic treated group compared with control group. In spirulina co-administered group, the size of endometrial glands and thickness of endometrium and myometrium were significantly restored to nearly normal control one (Table 1). Histomorpho-metrical examination of the serial sections of ovarian tissue showed that the exposure to sodium arsenate significantly decreased the number of ovarian follicles ($P<0.01$) in comparison with control group. Co-treatment

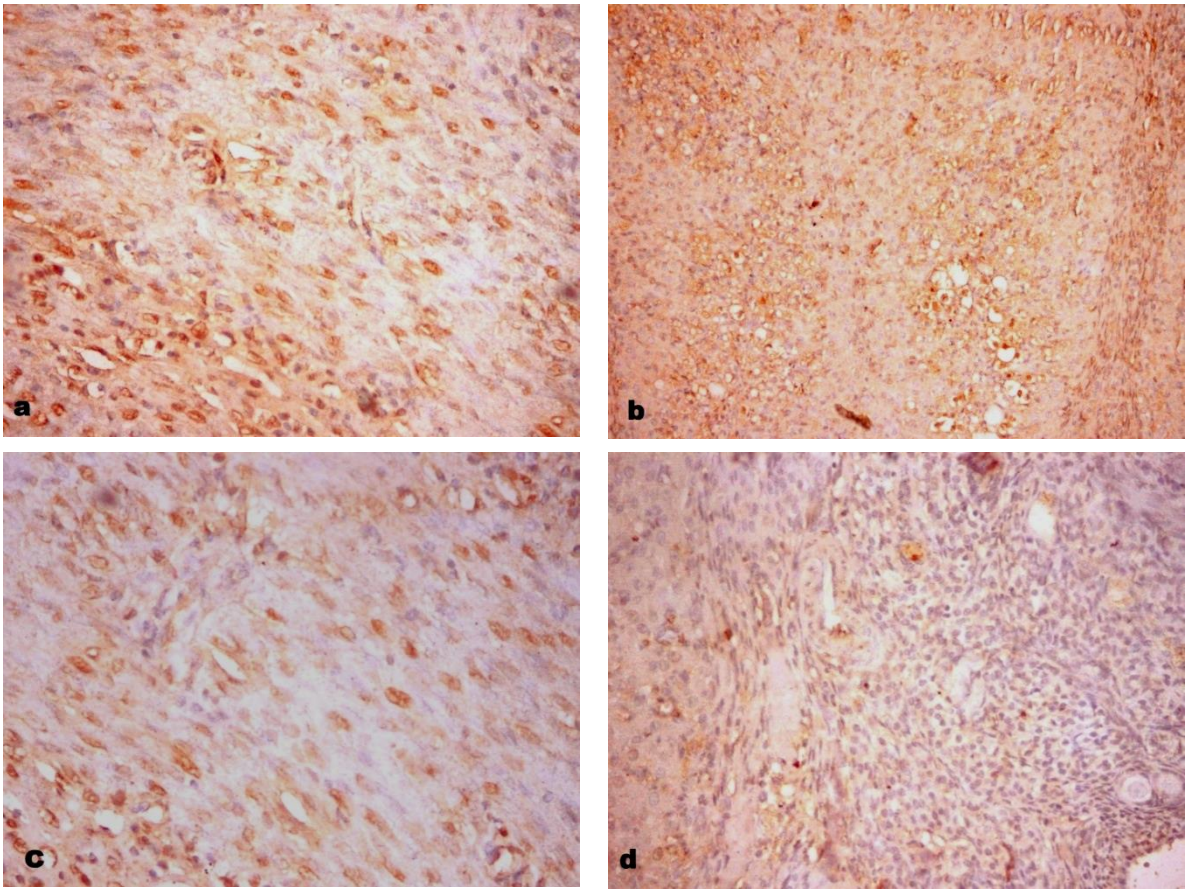

Fig. 5: Immunostaining of Nrf2. Arsenic treated group showing strong positive cytoplasmic reaction in stromal cells of uterus **(a)** and ovary **(b)**. In spirulina co-treated group, the immunostaining reaction is weak in both uterus **(c)** and ovary **(d). (H & E X400).**

with spirulina significantly increased the number of ovarian follicles compared to arsenic treated group (Table 2).

Immunohistochemistry

Nrf2 Expression

Nrf2 expression in uterus and ovary revealed an intense reaction in stromal cells of the uterus and the ovaries of As-treated group (Fig. 5a & b). This strong reaction shown with As-treated rats is abated to less intense reaction with the co-administration of *Spirulina* in the As + Sp group revealing the tendency to return to the control expression of Nrf2 (Fig. 5c & d).

DISCUSSION

Arsenic is a natural component of the environment and widely used in human activities. Thus, people are inevitable to contact with pollutant of arsenic along with the increase of arsenic in the environment (Zhao *et al*., 2017).Sodium arsenate is the pentavalent form of inorganic arsenic and is biotransformed quickly into sodium arsenite, a trivalent form of arsenic. Numerous reports have substantially shown that arsenic exposure irrespective of the inorganic salt form leads to a tremendous increase in the generation of free radicals (Firdausa *et al*., 2018). The naturally occurring sources of antioxidants have been used in various in vitro and in vivo studies and have demonstrated promising outcomes with regard to their effects on metal-induced toxicity (Khalil *et al*., 2018).

The present experiment was designed to assess the deleterious effect of Sodium arsenate (Na_3AsO_4) on the female reproductive organs (uterus and ovaries) in female albino rats following oral exposure, and to evaluate the efficacy of *Spirulina platensis* on modulating the female reproductive and neurotoxic alterations induced by Sodium arsenate with determination of its pathways of protection.

Effect of Sodium arsenate on animals' body weight has been studied by many researchers (Elshawarby *et al*. 2014; Khan *et al*., 2014 and Rodriguez *et al*., 2016). In this study, the adverse effect of As- induced toxicity on body weight was significantly ($P< 0.01$ and $P< 0.001$) present. As-treated female rats showed a significant decrease in body weight and retardation of growth in comparison with control group. Although (Souza *et al*. 2016) reported that arsenic exposure did not affect the total body weight in female rats in their study but this may be due to the dose or duration of their studies or may be the animal model used. Body weight of female As-treated rats was significantly recovered by spirulina co-administration. This improvement could be due to the unique nutrients that present in Sp, like B-complex vitamins, minerals, proteins, γ-linolenic acid and anti-oxidants like β-carotene, vitamin E (Holman and Malau-Aduli, 2012).

In this experiment, the serum antioxidant enzymes GSH and MDA were measured and the results revealed significant reduction in the GSH in As-treated rats while showed significant increase of MDA levels. The co-

administration of Sp showed significant reverse of these values towards the control levels. The changes in levels of various biochemical parameters in sodium arsenate-treated rats indicate various aspects of metabolism of animals. Sodium arsenate decreased the activities of antioxidant enzymes, thereby generating oxidative stress as evidenced by increased lipid peroxidation levels. The presence of antioxidant enzymes helps in combating free radicals/oxygen-derived species generated during normal physiological process. Any alterations in the activities of these enzymes change the redox status of the cells, thus altering the normal physiological processes. Arsenic exposure decreased the activities of antioxidant enzymes along with increase in lipid peroxidation. A continued oxidative stress as indicated by the increased MDA values usually causes inflammation, which in turn may lead to chronic diseases, including cancer (Mehta &Hundal, 2016).

Histopathologically, female reproductive system showed also degenerative changes in both uterus and ovaries in As-treated rats, and these findings collaborate with the previous findings of (Akram *et al*.,2010; Wares *et al*. 2013). Also,the size and number of endometrial glands and thickness of endometrium and myometrium were significantly reduced. Exposure to sodium arsenate significantly decreased the number of ovarian follicles, these changes could be attributed to that, arsenic is an endocrine disruptor influences sex hormones and induces inhibition of ovarian steroidogenesis and reproductive disturbances (Sun *et al*. 2016). It is known that arsenic treatment is also associated with the hypertrophy of adrenal gland, and inhibition in gonadotropin secretion (Chatterjee and Chatterji, 2011). The cellular degeneration of the female sex organs in arsenic-exposed rats may have resulted from the low levels of plasma gonadotrophins and estradiol as ovarian-cell proliferation and differentiation are controlled by gonadotrophins, while uterine weight and histoarchitecture are regulated by plasma estradiol level. Elevation in the number of atretic follicles and diminution in the numbers of healthy follicles may be due to low plasma levels of gonadotrophins and estradiol. In another way, these changes may be elucidated by arsenic-induced oxidative stress in ovary, which is supported by the diminution in the activities of GSH along with overproduction of MDA levels. (Chattopadhyay and Ghosh, 2010).Uterine endometrium degeneration is associated with the increased production of reactive oxygen species (ROS) such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Endometrium may be a potential candidate site for superoxide anion generation in the uterus because ROS are generated in the endometrium. Arsenic is known to produce oxidative stress by the promotion of ROS. Under normal conditions endometrial stroma regulates the growth and function of endometrial glands. Possibly disrupted endometrial stroma may affect the growth and differentiation of endometrial glands (Akram *et al*., 2010). Co-administration of Sp revealed a protection against these degenerative changes seen in the histopathology of As-treated rats and showed a normal uterine and ovarian structure.

Potency of spirulina may be attributed to c-phycocyanin, which may impact the function of mast cells which are a critical source for the synthesis and release of prostaglandin D2, reactive oxygen species (ROS), leukotrienes, and cytokines, such as TNF-α, which trigger the release of interleukin (IL)–1β, IL–6, as well as block the phosphorylation of p38 mitogen-activated protein kinases (MAPK), which in turn regulate the synthesis of cytokines. Furthermore, Spirulina diminishes nitrite generation, suppresses inducible nitric oxide synthase expression, and lessens liver microsomal lipid peroxidation (Khalil *et al*., 2018)

In this study, immunohistochemistry was used to prove the role of oxidative stress in As- induced toxicity and to assess the role of Sp in counteracting the arsenic mode of action and ameliorating the As-mediated oxidative stress in As- induced toxicity. The results revealed the overexpression of Nrf2 in the ovaries and uterus of As-treated rats and weak expression of Nrf2 with Sp co- treatment. Numerous studies have shown that arsenic was an Nrf2inducer in several cell types. Exposure to arsenic or other exogenous stressors may activate the Nrf2 pathway to maintain cellular redox homeostasis and limit oxidative damage, Oxidative stressactivatesNrf2 by permitting its dissociation from Keap1and translocation into the nucleus where it binds to the antioxidant response element and leads to the expression of the target genes (Li *et al*., 2015).

In conclusion, sodium arsenate induced reproductive toxicity in female albino rats could be ameliorated by *Spirulina platensis*co- treatment, with focusing on the role of Nrf2 in arsenic-induced toxicity as *Spirulina platensis* counteract the arsenic-induced oxidative stress through activating the Nrf2 pathway.

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