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Potential Alleviation of *Chlorella vulgaris* and *Zingiber officinale* on Lead-Induced Testicular Toxicity: an Ultrastructural Study

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Natural products were studied to combat reproductive alterations of lead. The current work aimed to disclose the efficacy of *Chlorella vulgaris* and *Zingiber officinale* to alleviate lead acetate induced toxicity. Sixty adult male Wistar rats were distributed into four groups. Group 1 was considered control, group 2 received 200 mg/l PbAc water, group 3 received 50 mg/kg/rat of *C. vulgaris* extract and 200 mg/l PbAc water, and group 4 received 100 mg/kg/rat of *Z. officinale* and 200 mg/l PbAc water for 90 days. Testis samples were subjected to ultrastructural examination. It was observed that PbAc caused degenerative alterations in the spermatogenic series in many tubules, with a loss of germ cells and vacuoles inside the cytoplasm and between the germ cells. Mitochondria exhibited ballooning, with lost cristae and widening of the interstitial tissue, while nuclear envelopes of primary spermatocytes were broken up, and axonemes of the mid-pieces of the sperms were distorted. With the treatment with *C. vulgaris* or *Z. officinale*, there were noticeable improvements in these modifications. It was concluded that both *C. vulgaris* and *Z. officinale* represent convincing medicinal components that may be used to ameliorate testicular toxicity in those exposed to lead in daily life with superior potentials revealed by *C. vulgaris* due to its chelating action.

Key words: *Chlorella vulgaris,* lead acetate, ultrastructure, *Zingiber officinale*.

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Exposure to lead (Pb) is considered a significant health problem (JACKIE *et al*. 2011). The toxicity of lead is principally related to oxidative stress through the creation of reactive oxygen species (ROS) like hydroxyl radicals, superoxide radicals, lipid peroxides, and hydrogen peroxide (PATRA *et al*. 2011).

The deterioration of reproductive abilities in the male is the main manifestation of occupational exposure to lead (KAKKAR & JAFFERY 2005). Diminished production of spermatozoa in terms of quality and amount has been observed after evaluation of workers of industrial facilities that rely on lead (NAHA *et al*. 2003). Contact with lead reduces the activities of testicular steroidogenic enzymes (CHEY & BUCHANAN 2008).

Chlorella vulgaris is a green unicellular algae that is used in chelating harmful metals (MEHTA & GAUR 2005). These microalgae have been used to retrive ions including nickel, chromium, cadmium, and lead from water. This is due to the existence of a metal-ion binding site affinity in these microorganisms (AKSU & DÖNMEZ 2006). This metal-binding capacity appears to be associated with the existence of chloroplasts within the cell wall, which is a cell organelle full of sulfur, potassium, calcium, and phosphorus (PEÑA-CASTRO *et al*. 2004). The capability of substances that contain sulfhydryl to chelate metals is well known, and may represent the key mechanism in the *in vitro* elimination of hevy metal toxins by C. *vulgaris* (YUN *et al*. 2011).

Medicinal plants have been essential for humans to fight illnesses from the dawn of civilization (NAYAK *et al*. 2011). *Zingiber officinale* (family Zingiberaceae), the scientific name for ginger plant rhizomes, is a preferred cooking ingredient and spice all over the world (BALIGA *et al*. 2012). The distinctive therapeutic components of ginger herb result from phytochemicals such as shogaols, gingerols, zingerone, zingiberene, and gingerdiol, recognized to have antioxidant properties (KHAKI & KHAKI 2010).

The aim of the current work is to investigate the ameliorative influences of *Chlorella vulgaris* and

Zingiber officinale for the alleviation of adverse consequences of exposure to lead.

Material and Methods

Ethical Approval

This study was conducted following the approval by the Medical Research Ethics Committee of Faculty of Medicine, King Abdulaziz University (Reference No 1167-13) on 9-9-2013.

Animals

Sixty male adult Wistar rats weighing 220±15 g were obtained from the animal house and randomly distributed into four groups (N=15). The rats were individually housed in stainless steel cages, and kept under standard conditions, including a controlled temperature of 20±2°C and humidity of 50%; the lighting cycle was 12 h light and 12 h dark, and appropriate ventilation was applied. Furthermore, the rats were fed with standard diet pellets with food and water ad libitum. Animals were handled in accordance with the guidelines of the National Institutes of Health.

Chemicals

Lead acetate trihydrate [$(C_2H_3O_2)_2Pb.3H_2O$] (PbAc) was purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). The *Z. officinale* root was obtained from the local market, cut into tiny parts, and dried for 6 days. The dried root was ground in a grinder and powder was obtained. Fifty grams of powder were extracted in 250 ml of ethanol for 18 h in a Soxhlet extractor, and then the extract was dried at low pressure and stored at 4°C (EL-SHARAKY *et al*. 2009).

Chlorella vulgaris Extract (CVE)

CV Beijerinck (strain 072), a strain of unicellular green algae was obtained from Medical supplier (Kuala Lumpur, Malaysia). CV algae were cultured in a tank in Bold Basal Media (BBM) outdoors with 12 h sun, 12 h dark and adequate aeration. Then CV algae were centrifuged 3 times at 3000 rpm for 10 minutes at 400°C to separate the media. Pelleted algae were diluted in distilled water at 150 mg/kg body weight before being used throughout the experiment.

Experimental Design

The duration of administration was 90 days. Group 1 was considered the control, and received distilled water. Group 2 received 200 mg/l of PbAc water. Group 3 received *C. vulgaris* extract at a dose 50 mg/kg/rat body mass and 200 mg/l of PbAc water (YUN *et al*. 2011). Group 4 received 100 mg/kg/rat of *Z. officinale* and 200 mg/l of PbAc water (ZAHEDI *et al*. 2010).

Testicular Histology

Testes were fixed in 10% neutral buffered formalin. For each specimen, at least three to five slides were stained with hematoxylin and eosin (H&E) and examined using an Olympus BX53 microscope equipped with a DP73 camera (Olympus, Tokyo, Japan).

Morphometry

At least 30 tubular profiles of each animal, i.e. round or almost round seminiferous tubules, were selected indiscriminately and analyzed. The mean seminiferous tubule diameter (MSTD) was obtained by measuring across the minor and major axes. The seminiferous epithelium height was measured for the same tubules; this was calculated as the space between the tunica propria and the edge of the lumen, and two diametrically opposed readings were taken with a digital ruler on each cross-section using their mean values (SHAYAKHMETOVA *et al*. 2013). The measurements were taken at different magnifications using Image-Pro Plus v6.0 (Media Cybernetics, Maryland, USA).

Ultrastructure

Testis samples of approximately 1 mm^3 were obtained and immersed in 2.5 % glutaraldehyde in 0.1 M buffer phosphate at 4°C for 3 h and post-fixed in 1% osmium tetraoxide. After dehydration in ascending grades of ethanol, the tissues were embedded in Epon 812. Semi-thin sections were prepared from the blocks, stained using toluidine blue, and demonstrative areas of semi-thin section were chosen. Ultrathin sections of 50-60 nm thickness were cut using an ultramicrotome (NOVA, LKB 2188, Bromma, Sweden). Following this, uranyl acetate and lead citrate were used to stain the tissues, which were then inspected with a Philips 201 transmission electron microscope at 60-80 kV in the Transmission Electron Microscope Unit (Philips Industries, Eindhoven, Netherlands) (MUSTAFA 2012; MUSTAFA *et al*. 2013).

Statistical Analysis

All data are presented as the mean ± standard deviation (SD) of different parameters between treated groups. Data evaluation was carried out using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Differences were considered significant if $P<0.05$. All statistical analyses were performed using SPSS software v22.

Results

Histological results

The testes of the control group showed the normal morphology of seminiferous tubules (Fig. 1A). The group treated with PbAc showed some shrunken spermatogenic cells with pyknotic nuclei and acidophilic hyaline homogenous material in interstitial tissue. Myoid cells were observed with flat nuclei in the basal lamina around the tubules. The interstitial Leydig cells appear rounded in shape with rounded vesicular nuclei. These cells are associated with small blood capillaries. Numerous vacuoles are apparent in the interstitial tissues and between the spermatogenic cells, and there are empty areas in-between the spermatogenic layer (Fig. 1B). The group treated with PbAc and *C. vulgaris* showed restoration of the architecture of seminiferous tubules and nearly healthy germ cells; the tubules are lined by Sertoli cells, primary spermatocytes, rounded spermatids, and elongated spermatids. The interstitial tissue contains interstitial Leydig cells (Fig. 1C). The group treated with PbAc and *Z. officinale* showed rebuilding of the spermatogenic series (Fig. 1D).

Morphometric results

There was a significant decline in the mean diameter of the seminiferous tubules in the lead acetate treated group (253.914±24.69) in comparison to the control group (273.262±64.87) (P=0.0001). In lead acetate treated with *C. vulgaris*, the mean diameter of the seminiferous tubules was insignificantly different from control (266.621±30.16) (P=0.058) but was significantly higher than in the lead acetate group (P=0.0001). In lead acetate treated with *Z. officinale*, the mean diameter was significantly lower than control (263.934±37.76) (P=0.002) but was significantly higher than in the lead acetate group (P=0.001) (Table 1).

There was a significant decline in the mean height of the germinal epithelium in the lead acetate treated group (57.367±10.88) in comparison to the control group (67.824±4.26) (P=0.0001). In lead acetate treated with *C. vulgaris*, the mean height of the germinal epithelium was insignificantly different from control (66.062±20.58) (P=0.126) but was significantly higher than in the lead acetate group (P=0.0001). In lead acetate treated with *Z. officinale*, the mean diameter was significantly lower than control (65.362±9.37) (P=0.012) but was significantly higher than in the lead acetate group (P=0.0001) (Table 1).

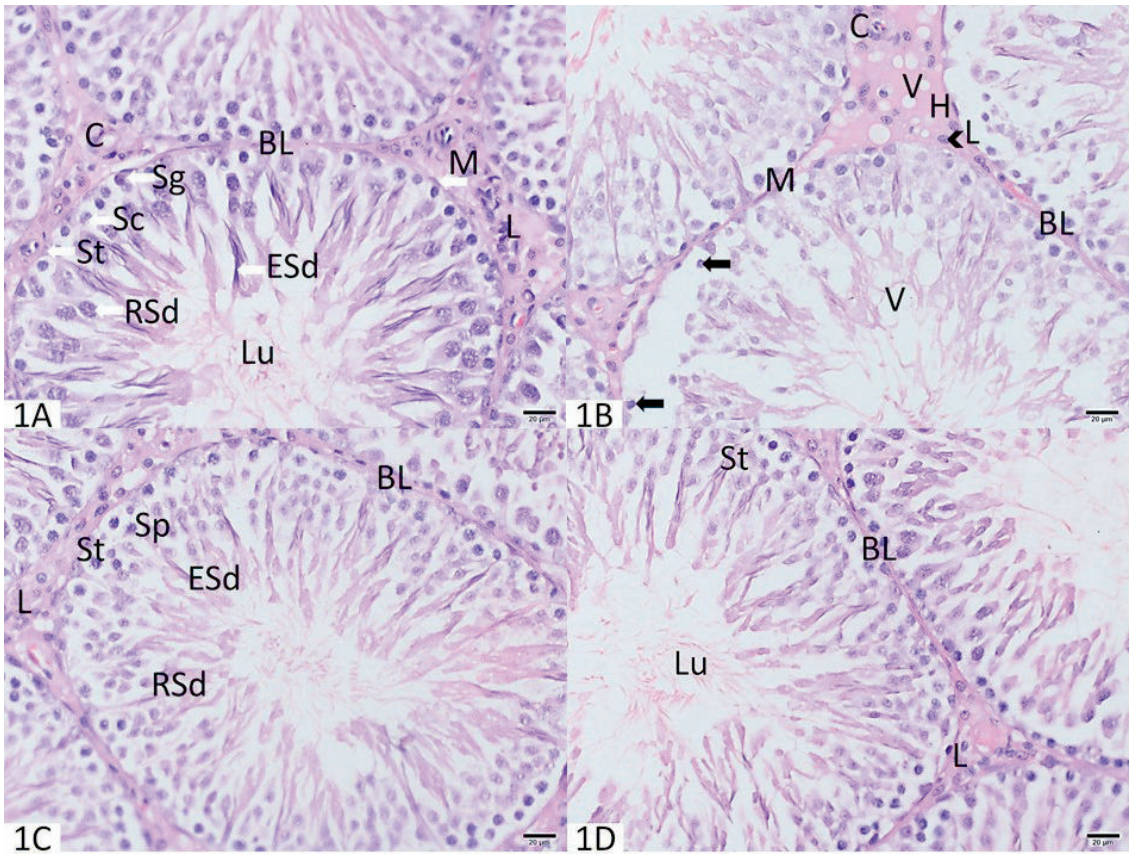

Fig. 1 A-D. 1A – Photomicrograph of a testis section from group 1 showing basal lamina (BL), myoid cells (M), Sertoli cells (St), spermatogonia (Sg), primary spermatocytes (Sc), rounded spermatids (RSd), elongated spermatids (ESd), tubular lumen (Lu), Leydig cells (L) and blood capillaries (C). 1B – Group 2 showing spermatogenic cells with shrunken pyknotic nuclei (arrow), hyaline material (H), and numerous vacuoles (V). 1C – Group 3 showing rebuilding of the spermatogenic layers. 1D – Group 4 showing restoration of the spermatogenic series (Sp). (H&E. Bar = 20 μm).

Table 1

Mean diameter of seminiferous tubules and germinal epithelial height by µm

	Control (N=200)	Lead acetate (N=200)	Lead acetate + *C. vulgaris* (N=200)	Lead acetate + *Z. officinale* (N=200)
Tubular diameter (MSTD) (µm)	273.262±64.87	253.914±24.69 [1]P<0.0001*	266.621±30.16 [1]P<0.058 [2]P<0.0001**	263.934±37.76 [1]P<0.002* [2]P<0.001**
Germinal epithelium height (µm)	67.824±4.26	57.367±10.88 [1]P<0.0001*	66.062±20.58 [1]P<0.126 [2]P<0.0001**	65.362±9.37 [1]P<0.012* [2]P<0.0001**

Values are expressed as a mean ± SD. The analysis was made using one-way ANOVA followed by Tukey's post hoc test. [1]P(*): significance versus control; [2]P(**): significance versus lead acetate group.

Results for semithin sections

The control group showed a normal architecture (Figs 2A and 3A). The group treated with PbAc showed depletion of the germ cells, with many vacuoles (Fig. 2B), and interstitial Leydig cells revealed a vacuolated cytoplasm (Fig. 3B). The group treated with PbAc and *C. vulgaris* showed a nearly healthy spermatogenic series (Fig. 2C and 3C). The group treated with PbAc and *Z. officinale* showed restoration of spermatogenic cells, with minute vacuoles still detected (Fig. 2D and 3D).

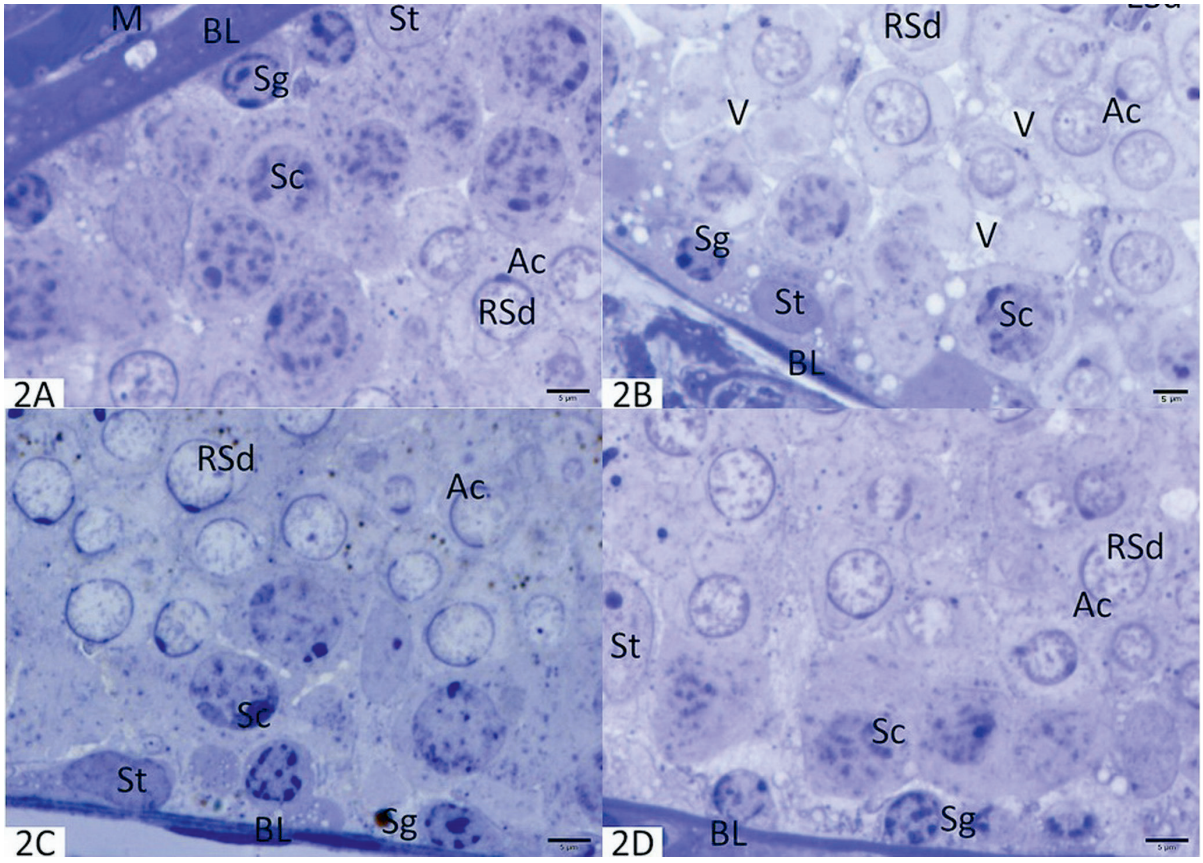

Fig. 2 A-D. 2A – Photomicrograph of a testis section from group 1 showing a spermatogenic epithelium formed of spermatogonia (Sg) with mitotic figures, primary spermatocytes (Sc), and round spermatids (RSd) with acrosomal caps (Ac). Note also Sertoli cells (St) with characteristic indentation of the nucleus and myoid cells (M) just outside the basal lamina (BL). 2B – Group 2 shows depletion and separation of the spermatogenic cells with vacuoles (V) and empty spaces between and inside the spermatogenic cells. Note elongated spermatids (ESd). 2C – Group 3 shows healthy spermatogenic series. 2D – Group 4 shows restoration of the spermatogenic cells. (Toluidine Blue. Bar = 5 µm).

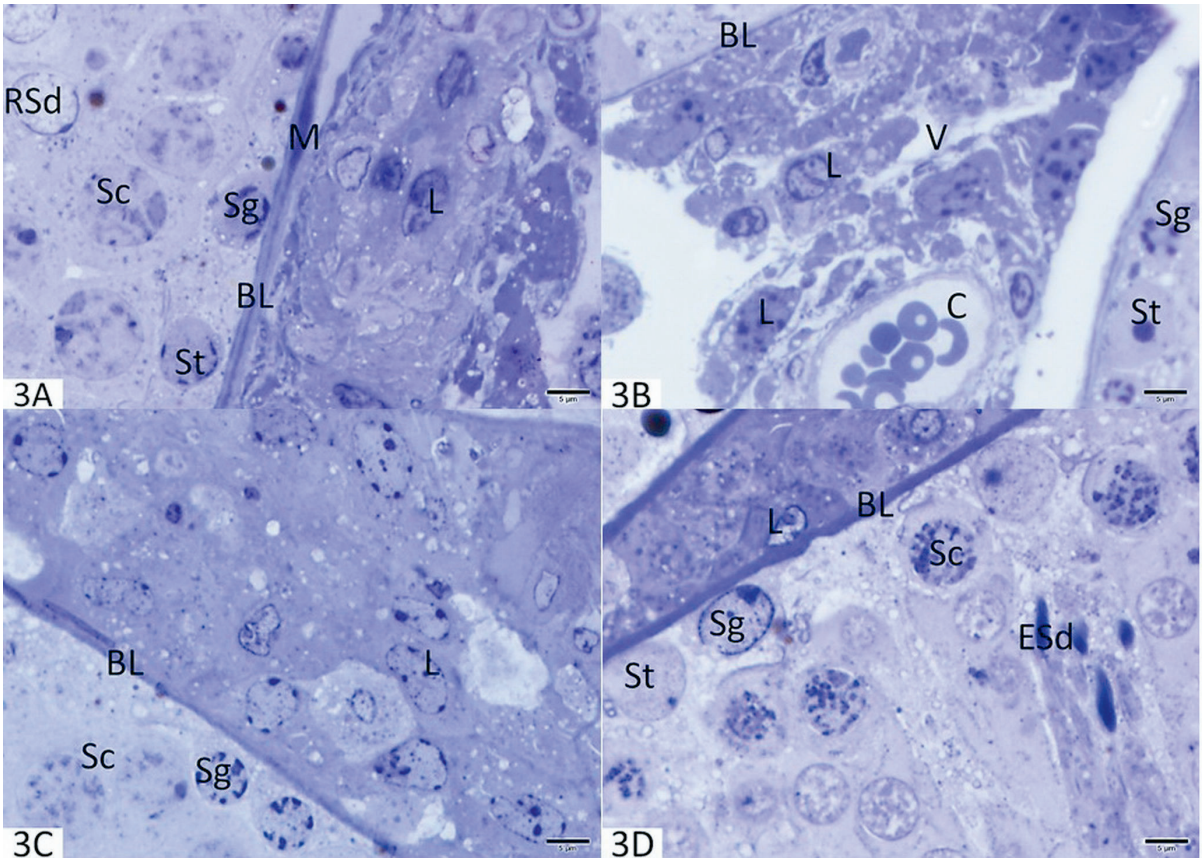

Fig. 3 A-D. 3A – Photomicrograph of a testis section from group 1 showing interstitial space containing Leydig cells (L). Note spermatogonia (Sg), primary spermatocytes (Sc), round spermatids (RSd), acrosomal caps (Ac), Sertoli cells (St), myoid cells (M), and basal lamina (BL). 3B – Group 2 shows wide interstitial spaces with many interstitial cells of Leydig (L), and a vacuolated cytoplasm (V). Note the large congested blood vessel (C). 3C – Group 3 shows almost healthy architecture. 3D – Group 4 shows restoration of interstitial Leydig cells (L) with minimal vacuoles. (Toluidine Blue. Bar = 5 µm.)

Ultrastructural results

The group treated with PbAc showed an irregular nucleus of Sertoli with a characteristic indented nucleus resting on the thickened basal lamina containing collagen and vacuolated, distorted mitochondria and well-noticeable vacuoles of variable sizes. In addition, disintegrated nuclear envelopes of primary spermatocytes were observed with vacuolated, distorted mitochondria having damaged cristae and many well-defined vacuoles covering the cytoplasm. Moreover, the intact zone of tight junctions between the Sertoli cells and dilated smooth endoplasmic reticulum (sER) were observed (Fig. 4A). Spermatids showed damaged chromatin and the mid-pieces of the sperm exhibited marked distortion of the axoneme (Fig. 4B and 4C). Leydig cells had an irregular nucleus and peripheral heterochromatin (Fig. 4D). The group treated with PbAc and *C. vulgaris* showed preserved histological structure of most of the seminiferous tubules; some mitochondria were affected, while others were healthy. Moreover, empty spaces were noticed throughout (Fig. 5A and 5B). The central axonemes of the mid-pieces of sperm were apparently healthy (Fig. 5C). Leydig cells had irregular euchromatic nuclei and the cytoplasm contained electron-dense bodies of variable sizes (Fig. 5D). The group treated with PbAc and *Z. officinale* showed nearly healthy seminiferous tubules, but numerous unhealthy mitochondria with lost cristae and intact zones of tight junctions (Fig. 6A). Moreover, few empty spaces in the cytoplasm were observed (Fig. 6B). The mid-pieces of the sperm showed distortion of the mitochondrial sheaths (Fig. 6C). Leydig cells exhibited euchromatic nuclei and some electron-dense bodies (Fig. 6D).

Discussion

In the present work, the PbAc-treated group revealed reduction in the histological architecture, which agreed with a previous study (ABDEL MONEIM *et al.* 2011). The probable mechanism for this damage is the direct effect of lead on the gonads, the hypothalamic-pituitary axis, or both, which can suppress spermatogenesis (MABROUK

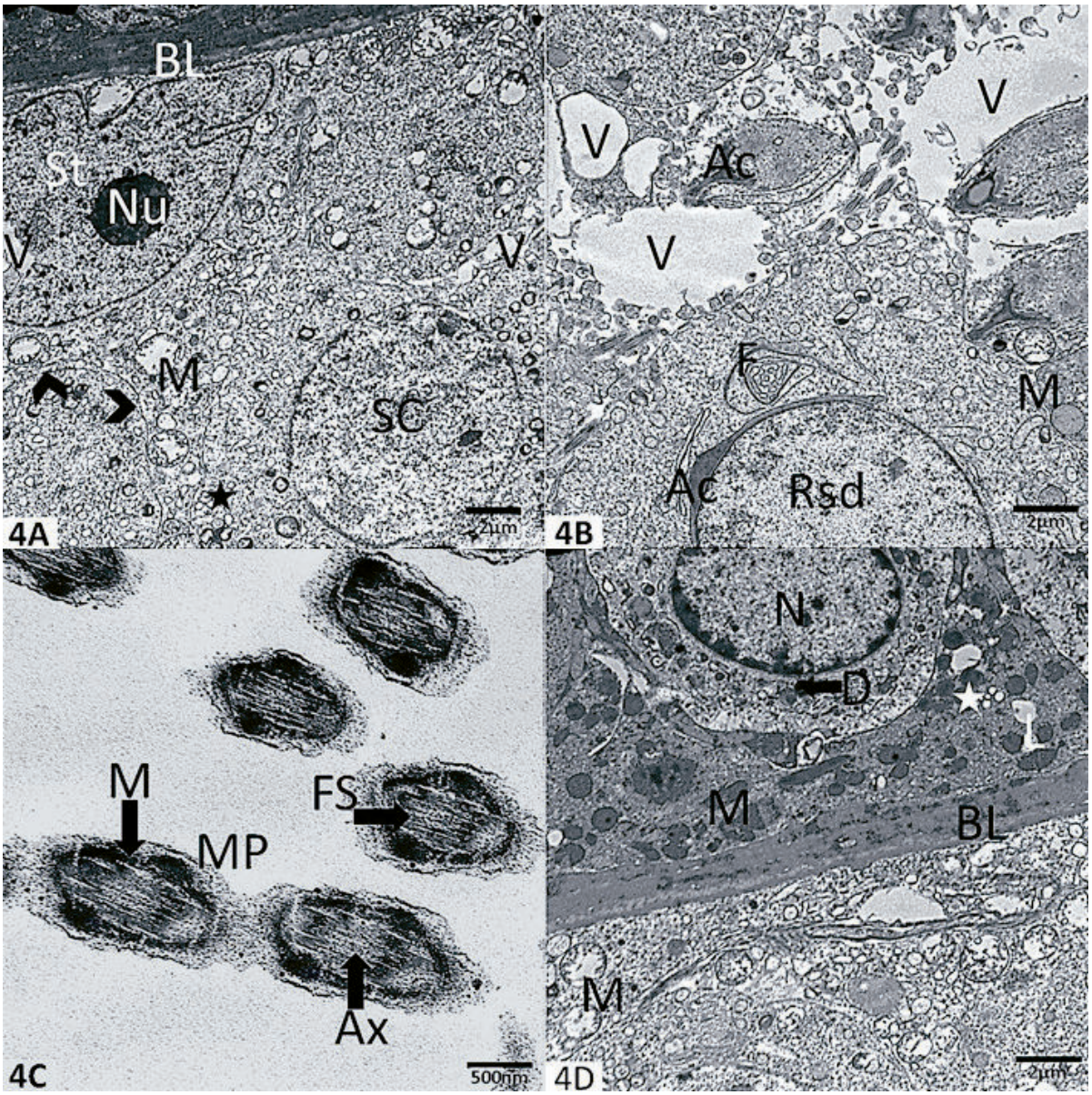

Fig. 4 A-D. 4A – An electron micrograph of a testis from group 2 showing a Sertoli cell (St), basal lamina (BL), primary spermatocytes (Sc), mitochondria (M), vacuoles (V), zone of tight junctions (arrowhead) and smooth endoplasmic reticulum (star). 4B – Rounded spermatids (Rsd) with rounded or oval nuclei that have damaged chromatin; peripherally arranged mitochondria with lost cristae (M) and the characteristic acrosomal cap (Ac) can be observed. Note the myelin-like figure (F) and numerous empty spaces (V) throughout. 4C – Cross-sections of mid-pieces (MP) of the sperm, with distortion of the central axoneme (Ax), mitochondrial sheath (M), and fibrous sheath (FS). 4D – Leydig cells near the basal lamina (BL) with irregular euchromatic nucleus (N) and peripheral heterochromatin. Electron-dense bodies of variable sizes (D), mitochondria (M), dilated sER (star), and lipid droplets (L) are noticed. Note that for Fig. 4A, 4B, and 4D, the scale bar is 2 µm; for Fig. 4C, it is 500 nm.

& CHEIKH 2014). In addition, it could be attributed to excessive production of free radicals and lipid peroxidation (ESKENAZI *et al*. 2005). Shrinkage in the seminiferous tubules is caused by contraction of myoid cells (GARCIA-LESTON *et al*. 2010).

In the current research, many seminiferous tubules were found devoid of germ cells; this may be due to the retraction of the cytoplasmic processes of Sertoli cells, which support germ cells. It may also affect the division and differentiation of spermatogonia and cause apoptosis (SAWHNEY *et al*. 2005). In addition, it might be attributed to a reduction in testosterone caused by lead, leading to disruption of Sertoli junctions (LIU *et al*. 2003).

In the current work, minimal changes were observed in the basal lamina thickness. This is against previous results, showing an increase in basal lamina thickness in the PbAc-treated group and attributed this thickening to an increase in the collagenous fibers, either due to the overproduction of collagen by fibroblasts or due to a decrease in collagen phagocytosis (GIULIANI *et al*. 2005).

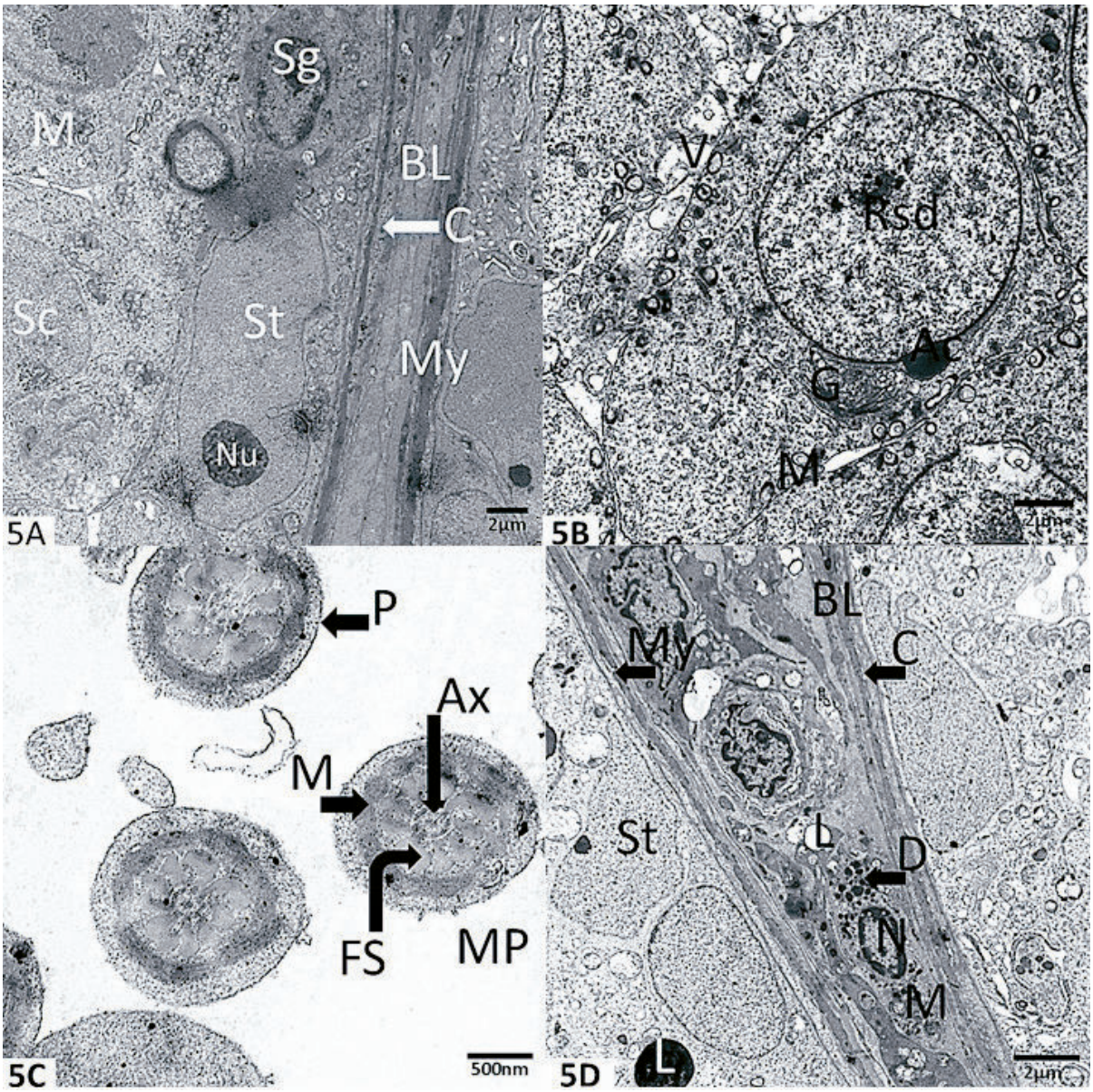

Fig. 5 A-D. 5A – An electron micrograph of a testis from group 3 that showed a Sertoli cell (St) with a euchromatic-indented nucleus and apparent nucleolus (N) and the spermatogonia (Sg) resting on the thickened basal lamina (BL) with a myoid cell (My) and collagen (C). Some mitochondria are affected, while others are healthy (M). 5B – Rounded spermatids (Rsd) with peripherally arranged unhealthy mitochondria (M) and the characteristic acrosomal cap (Ac) enwrapped by Golgi saccules (G). Note that there are few empty spaces (V) in the cytoplasm. 5C – Cross-sections in the mid-pieces (MP) of the sperms that have a central axoneme (Ax) surrounded by a mitochondrial sheath (M), a fibrous sheath (FS), and a peripheral cell membrane (P). 5D – Leydig cells with irregular euchromatic nucleus (N) and peripheral heterochromatin. Electron-dense bodies of variable sizes (D), mitochondria (M), and lipid droplets (L) are present in the cytoplasm. Note that for Fig. 5A, 5B, and 5D, the scale bar is 2 µm; for Fig. 5C, it is 500 nm.

The junctions in-between Sertoli cells in the PbAc-treated group were found to remain intact in the present work, which may be attributed to the resistance of these junctions to lead or the effects of paracrine hormone from the nearby Leydig cells (MARCHLEWICZ *et al*. 2004).

In the present work, the wide interstitial spaces with numerous vacuoles in the interstitial Leydig cells could be explained by degeneration of these cells and reduction in testosterone, which are associated with spermatogenic arrest (MAKHLOUF *et al*. 2008). Against that view, the hypercellularity of Leydig cells may occur as a compensatory process secondary to feedback to the pituitary gland due to a decline in testosterone (JENSEN *et al*. 2006). In the existing findings, the smooth endoplasmic reticulum (sER) revealed dilated cisternae; this can be explained according to testosterone entrapment in these cisternae (EL SHAFAI *et al*. 2011). Contradicting this, others observed a reduction of the surface area of sER in the Leydig cells in a number of PbAc-treated groups (RUBIO *et al*. 2006).

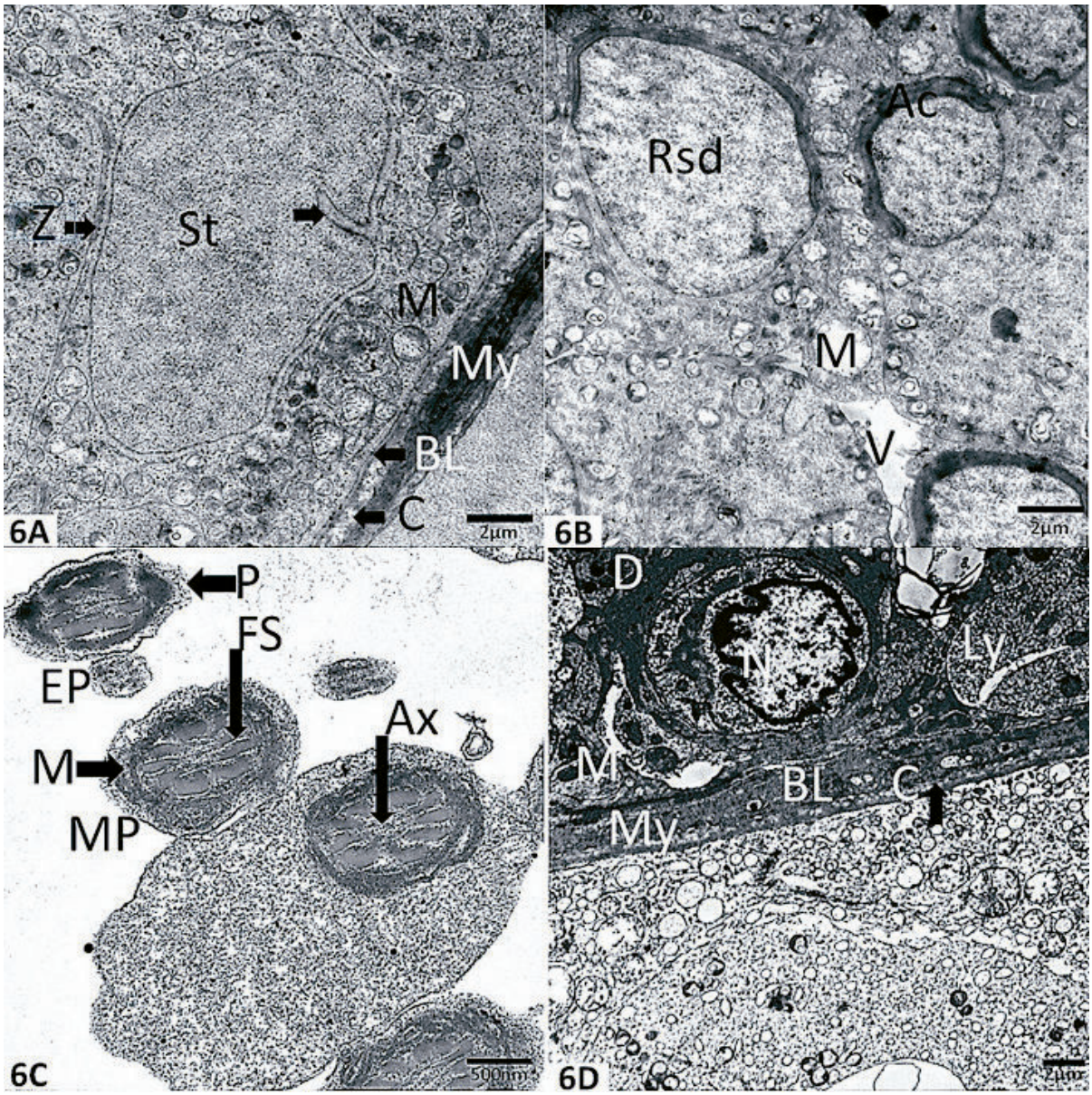

Fig. 6 A-D. 6A – An electron micrograph of a testis from group 4 showing a Sertoli cell (St) with a euchromatic-indented nucleus (arrow) resting on the thickened basal lamina (BL) with a myoid cell (My) and collagen (C). Note the intact zone of tight junctions (Z) and numerous unhealthy mitochondria with lost cristae (M). 6B – Rounded spermatids (Rsd) with the characteristic acrosomal cap (Ac) and peripherally arranged unhealthy mitochondria (M) can be observed. Note some empty spaces (V) throughout. 6C – Cross-sections of mid-pieces (MP) of the sperms with minimal distortion of the mitochondrial sheaths (M) and fibrous sheath (FS). Note that the end-pieces (EP) have a central axoneme (Ax) surrounded by outer cell membranes (P). 6D – Leydig cells with irregular euchromatic nuclei (N) and external heterochromatin. Variable-sized electron-dense bodies (D), mitochondria (M), and lymph (Ly) are observed in the cytoplasm. Note that for Fig. 6A, 6B, and 6D, the scale bar is 2 µm; for Fig. 6C, it is 500 nm.

Mitochondria were vacuolated and deformed in the current work; this is due to ROS that disturb the mitochondria through the inhibition of steroidogenic acute regulatory protein (StAR) expression (HUANG & LIU 2004). This also may result from apoptosis or an exposure of intracellular organelles to free radicals, which are ameliorated by free radical scavengers (WAKABAYASHI 2002).

Previous researchers observed an improvement in the number of spermatogenic cells and spermatozoa in goats treated with antioxidants (HONG *et al*. 2009). In the current work, treating PbAc-treated groups with *C. vulgaris* or *Z. officinale* revealed a rise in spermatozoa number in the lumen and rebuilding of the seminiferous epithelium.

C. vulgaris provides remarkable defensive mechanisms against PbAc-induced injury by eliminating heavy metal through the digestive function, either affecting absorption or excretion. When dietary *C. vulgaris* is given to animals with heavy metal, this stimulates toxicant excretion in the urine and feces (MIRANDA *et al*. 2001). *C. vulgaris* that is also known as *Parachlorella beyerinckii* (PB), prevented PbAc absorption from the digestive system with a significant increase in the amount of fecal excretion of lead (UCHIKAWA *et al*. 2009). Moreover, dietary fibers, which constitute approximately 10% of *C. vulgaris*, may be used to treat humans exposed to lead (WRIGHT *et al*. 2003).

QUEIROZ *et al*. noted that the administration of *C. vulgaris* hot-water extract (CVE) and pepsin-digestible proteins reduced the blood lead level in PbAc-treated groups, which is attributable to the chelating influence of the sulfhydryl-containing elements in CVE (QUEIROZ *et al*. 2003). As an additional factor, the mineral elements of BP, including iron, zinc, calcium, phosphorus, and magnesium may prevent lead absorption from the alimentary tract, probably through competition for shared absorptive receptors in the intestinal mucosa (WRIGHT *et al*. 2003).

The defensive action of *Z. officinale* is due to its content of volatile oils which have immunomodulatory and anti-inflammatory outcomes that can guard against testicular damage caused by the inflammatory process (MANNEM 2014). These volatile oils are able to suppress T-lymphocyte-dependent immune reactions (ZHOU *et al*. 2006). Furthermore, the anti-inflammatory action of *Z. officinale* is due to components including gingerol analogs (shogaols and paradols) (KHAKI & KHAKI 2010). Previous studies have noted that these ingredients are capable of inhibiting prostaglandin and leukotriene synthesis immediately (NURTJAHJA-TJENDRAPUTRA *et al*. 2003).

In the current findings, there was a persistent number of degenerative alterations in seminiferous tubules, many vacuolated germ cells, and damaged Sertoli cells in groups treated with PbAc and *C. vulgaris* or *Z. officinale*; this can be explained by the fact that the defensive capability of these substances against PbAc rely on the duration of exposure (QUEIROZ & WAISSMANN 2006). An additional reason is that these changes can be related to the overproduction of ROS by PbAc, which overcomes the cell's intrinsic antioxidant defense (HSU & GUO 2002). Furthermore, lead accumulates inside the cell nucleus and assaults nuclear protein and chromatin modifying their composition (TELIŠMAN *et al*. 2007).

The results of the presented experiments clarify the chelating and antioxidant effects on the ultrastructural changes of lead-induced toxicity in rat testes. The chelating effect of *Chlorella vulgaris* extract gave better results as regards the diameter of seminiferous tubules and germinal epithelium thickness, and sperm structure. The antioxidant effects of *Zingibar officinale* extract was less pronounced.

Conclusion

Chlorella vulgaris and *Z. officinale* can be recommended as an adjunct therapeutic strategy to combat lead-induced testicular damage. *C. vulgaris* showed superior potential through its antioxidant ability and chelating properties of heavy metals.

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