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Immunohistochemical Expression of Cytokeratin 18 in Rat Sertoli Cells under Effect of Aluminium Hydroxide and Pomegranate Molasses

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Abstract

Background: Sertoli cells are one of the major cell types in testes that provide nutrition and structural support for germ cell development. Cytokeratin 18 protein is consistently expressed in immature Sertoli cells of prepubertal seminiferous tubules, but is completely absent in normal, mature seminiferous tubules.

Objective: The objective of this study was to examine the possible protective role of pomegranate molasses. Through immunohistochemical expression of cytokeratin 18 in the rat sertoli cells of prepubertal age after the administration of aluminum hydroxide.

Materials and methods: Sixty male albino rats of prepubertal age were divided into three equal groups: (control group); (Aluminum hydroxide treated group) received oral aluminum hydroxide at a dose of 30 mg/kg b. wt/day; (protected group) received both aluminum hydroxide (at the same previous dose) and pomegranate molesses in a dose of 0.5 ml (PM) plus 0.5 ml distilled water orally. At the end of the experiment (8 weeks), all animals were sacrificed and their testes were excised. Paraffin sections were prepared for examing under the light microscope, used histomorphological stain and immunohistochemical DAB stain of Cytockeratin protein 18 by aviden biotin complex protocol.

Results: After the administration of aluminum hydroxide (AD), some seminiferous tubules had disturbed basal lamina and disorganized germinal epithelium. The spermatogenic cells decreased in number with undifferentiated hyperprolifirating germ cells. The concomitant administration of pomegranate molasses with aluminum hydroxide showed alleviation in histopathological changes induced by aluminum hydroxide in the structures of testis. In addition, the increased number of sertoli cells was noticed in grouped treated with AD and decreased in group treated with concomitant administration of PM.

Conclusion: The present study showed that exposure to aluminum hydroxide resulted in marked degenerative effects on the rats' testis, but they were improved with the concomitant administration of PM, as well as, the immunohistochemical detection of cytokeratin 18 in Sertoli cells may be provided a sensitive marker for immature or damaged testes.

Keywords: Aluminum hydroxide • Pomegranate molasses • Cytokeratin 18

Introduction

The male reproductive system may be affected seriously by metals directly, when they target specific reproductive organs, or indirectly, when they affect the neuroendocrine system. Metals have been shown to influence spermatogenesis in rodents and human, which can lead to abnormal sperm morphology, low sperm count and poor semen quality [1].

Aluminium (AL) is the third most abundant component in the earth's crust after oxygen and silicon, it is the most widely distributed metal in nature and is widely utilized as a part of day by day life that gives easy exposure to individuals [2].

AL enters the body through two main routes: pulmonary and oral. Aluminosilicates represent the bulk of inhaled contaminants in the air. Oral intake represents the route with greatest toxicological implications although only a small portion of AL is absorbed by the gastrointestinal tract [3]. The presence of aluminium chloride (ALCL) in drinking water has a suppressive effect on the fertility of male rat, also in male mice treated intraperitoneally with aluminum nitrate, necrosis of spermatocytes and a significant decrease in fertility were demonstrated [4,5].

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Pomegranate fruit, peel and juice have a marked antioxidant capacity because it has a high content of polyphenols. anthocyanins, catechins, condensed tannins, ellagitannins, gallic and ellagic acids. Pomegranate juice is also rich in vitamins and minerals, for example, vitamins E, C and B, iron, potassium and calcium [6].

Cytokeratins are intermediate filaments that represent an excellent marker for epithelial differentiation because all epithelial cells, whether of ectodermal, mesodermal, or endodermal origin, contain cytokeratins; Cytokeratin-18 (CK-18) and vimentin are typically co-expressed in the sertoli cell cytoplasm of the male fetus and additionally all through childhood. The relationship between abnormal Sertoli cell differentiation and spermatogenic impairment lead to examine the expression of Sertoli cell markers normally lost at puberty as CK18 [7].

Cytokeratin expression has been observed in adult Sertoli cells in testis with mixed atrophy associated with spermatogenic arrest at the level of spermatogonia [8]. In separate biopsies of individual testes with mixed tubular atrophy, this pathological condition found variable patterns of CK18 expression in Sertoli cells in adult men exhibiting various degrees of spermatogenic dysfunction as well as carcinoma *in situ* (CIS) cells [9].

The normal maturation of the sertoli cells is characterized by the absence of cytokeratin filaments, making it different from the other true epithelia. Expression of cytokeratin in adult seminiferous epithelium is regarded as a sign of either maintaining or regaining undifferentiated immature features. For example, testicular disorders resulting in infertility are associated with the presence of CK18 in sertoli cells. This may be a primary event indicating a failure of maturation of sertoli cells or a reversion of sertoli cells to a less differentiated state because of the absence of normal spermatogenic progression [7,9].

There are many studies carried on toxic effect of AL on the animal testis with the protective effect of variety of chemical agents, but this study was done to evaluate the role of PM as a protective agent against AL through histomorphological changes on rat testis as well as the maturation of Sertoli cells.

Materials and Methods

Materials

Aluminum hydroxide (ALOH) 500 g container was obtained from Al Gomhouria Company, Alexandria. Pomegranate molasses (PM) 250 ml bottle of 10% pomegranate juice concentrate from Kemal Kukrer Company; Turkey was obtained from a local market. Polyclonal Anti-Cytokeratin 18 antibody (CK18 antibody) IgG isotype, from rabbit host, reactive to human, mouse and rat was obtained from SNF Medical Company, Cairo.

The study was carried on sixty healthy male albino rats obtained and approved by guidelines for care and use of animals from the animal house of Faculty of Medicine, Alexandria University, Egypt (30 ± 2 days) age (prepupertal age).

The animals were maintained under standard laboratory conditions of temperature and humidity and 12 hour light/dark cycle. All rats were examined for health status and acclimatized for two weeks before the experiment. They were allowed free access of food (ad libitum) throughout the study.

After two weeks of acclimatization, these animals of 30 ± 2 days age (one month age as prepupertal age) were divided randomly into three equal groups as following: Group I (control group) included twenty male albino rats receiving normal diet and water ad libitum. Group II (ALOH treated): included twenty male albino rats receiving normal diet and ALOH in a dose of 30 mg/kg body weight orally for 8 weeks daily.

It was prepared immediately before use by dissolving it in distilled water and Group III (ALOH and PM treated): included twenty male albino rats receiving normal diet, ALOH at the same dose and duration as group II in addition to PM in a dose of 0.5 ml plus 0.5 ml distilled water orally at the same time and duration as ALOH. At the end of the study (8 weeks), the rats became at the age of puberty and sexually mature (three months old), twenty rats from each group were sacrificed under anesthesia and dissected to remove both testes.

Methods

Histopathological methods: The live testes organ was examined under the Stereomicroscope and the images of the control and experimental testes were picked up at 6X magnification. Histomorphometric studies were demonstrated on the image and illustrated the thickness of tunica albuginia and surface area of seminiferous tubules.

The testes were cut and fixed in 10% formalin for procesding the paraffin sections. Paraffin sections were mounted on clean slides, placed at 37°C oven. Sections were deparaffinized in xylene, rehydrated in descending grades of alcohol to distilled water, stained with Hematoxylin, washed in tape water then stained with Eosin, dehydrated in ascending grades of alcohol and cleared in xylene. Cover slip applied by Canada balsam and examined under light microscope and the images were picked up at the 40X magnification.

Immunohistochemichal detection of CK-18: Immunohistochemichal detection of CK-18 protein was used Avidin – biotin complex (ABC) protocol with the primary monoclonal antibody of CK-18 and observed under light microscope [10].

Paraffin sections were deparaffinized in xylene, rehydrated in descending grades of alcohol, then the sections were placed in 3% H_2O_2 and washed in phosphate buffer saline PBS. Then treated with antigen retrieval reagent using citrate buffer PH 6.5, the section treated with serum blocking agent (bovine serum albumin). Then the Sections incubated with the primary antibody at 4°C overnight in humidity chamber. A negative control sections were incubated without adding the primary antibody.

After washed the sections with PBS incubated with the biotinylated secondary antibody in humidity chamber at room temperature and incubated with ABC, then promoted with horse radish peroxidase, after washed by PBS the section stained by DAB chromogen and washed by distilled water and counter stained with Hematoxylin (nuclear counter stain) then washed in running tap water for 2-5 min, dehydrated with ascending alcohol series, cleared in xylene and mounted with DPX mounting media (mixture of Distyrene, plasticizer

and xylene) for observation under the light microscope and the images were picked up with 40X magnification [10].

Image analysis study: This was carried out using digital image analyzer using Olympus microscope – equipped with Spot digital camera, using computer program MATLAB software [11]. Ten images for different 5 slides for each group were recorded to evaluate the following:

a. Thickness of tunica albuginia (image from stereomicroscope, X6.5 magnification)

b. Surface area of seminiferous tubules (image from stereomicroscope, X6.5 magnification)

c. Sertoli cell count (image from light microscope, X400 magnification)

d. Image optical density (IOD): The measurement of the image optical density (IOD) changes of CK 18 immunohistochemical stain and trichrome stain density in the animal groups. The maximum, minimum and integrity of intensity color based on Gray-level acquisition, analysis of the data was carried out by the mean values immunostaining were based on the mean of pixel number. The IOD based on Gray-level transition probabilities in digitalized images from dark to light (0 up to 250).

Statistical analysis of data was fed to the computer and analyzed using IBM SPSS software package version 20.0 (Armonk, NY: IBM Corp) [12].

Results

Histopathological results

Paraffin section microphotographs of control rats testes (group I) showed normal architecture of seminiferous tubules appearing with regular outlines and surrounded by well-defined basal laminae. They were separated from each other's by interstitial tissue containing little connective tissue, blood vessels and clusters of interstitial cells of leydig that were polygonal in shape.

They were lined by multiple layers of the stratified germinal epithelium consisted of Sertoli and spermatogenic cells, at different stages of spermatogenesis. The high power image illustrated that the Sertoli cells identified by their typical irregular large pale nuclei and scattered at intervals between the spermatogenic cells.

Spermatogonia were oval in shape forming the basal cell layer, and the next layer was occupied by the primary spermatocytes that were spherical in shape with copious cytoplasm and large nuclei. Spermatids with rounded nuclei and spermatozoa were seen occupying the lumen of the tubules (Figures 1A(X400) and 1B(X1000)).

Photomicrograph of rat testes sections administrated aluminium hydroxide (group II) showed the semineferous tubules lost its characteristic architecture, had disturbed basal lamina and disorganized germinal epithelium. They were separated from the basal lamina and had multiple intercellular spaces due to germ cells desquamation.

The spermatogenic cells were hyperprolifirating undifferentiated germ cells with deeply stained nuclei that were nonfunctioning. The necrotic spermatids and wideness of the tubular lumen appeared with few sperms (maturation arrest). Dilatation of the interstitial blood vessels and interstitial vacuolation were noticed (Figure 1C (X400)).

Histopathological changes in rat testes administrated the aluminum hydroxide and pomegranate molesses (group III) showed restorement of the normal shape of the seminiferous tubules with intact basal laminae. The germinal epithelium became well stratified and well organized with restorement of the differentiated spermatogenic cells with abundant free spermatozoa in the lumen. Improvement of Interstitial Tissue (IT) in between the tubules was also observed (Figure 1D).

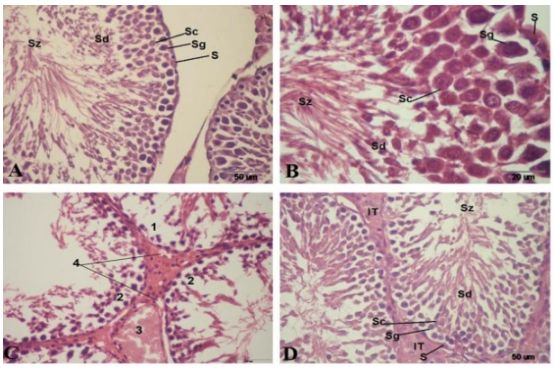

Figure 1. (A-D) Histopathological results in Paraffin section microphotographs of control rats testes (in various resolutions).

Immunohistochemical results

Paraffin sections stained with polyclonal CK18 antibody used avidin biotin complex and DAB chromogen in all groups. The immunohistochemical staining appeared as brown granules stained in the immature sertoli cells of control and experimental groups.

Cytokeratin 18 positive sertoli cells were few and localized at the periphery of the seminiferous tubules in both control group (Figures 2A and 2B). In group (II) there was increased number of hyperproliferating CK 18-positive Sertoli cells per tubule with increase of immunoreactivity (Figure 2C). In group (III) there was considerable recovery with decrease in the number of CK 18-positive Sertoli cells with decrease of immunoreactivity (Figure 2D).

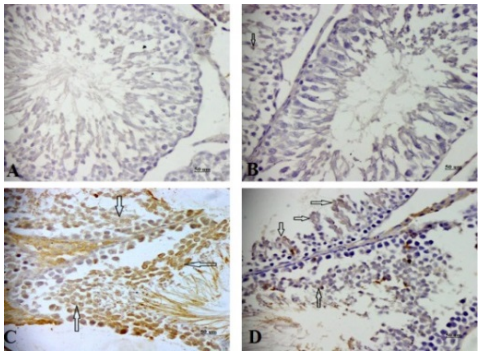

Figure 2. (A-D) Immunohistochemical results appeared as brown granules stained in the immature sertoli cells of control and experimental groups.

Image analysis results

Histomorphometry:

Thickness of tunica albuginea: Illustrated the thickness of tunica albuginea in the three studied groups. The mean level in control group was 2.86 ± 0.50, in group II (ALOH) was 91.59 ± 9.18 and in group III (ALOH & PM) was 42.21 ± 7.30. There was a significant increase in group II as compared to the control group, a significant decrease in group III as compared to the group II. There was a significant difference between the three studied groups regarding the thickness of tunica albuginea (p<0.001) (Table 1).

Table 1. Comparison between the three studied groups according to thickness of tunica albuginea.

Thickness of tunica albuginea	Control (n=20)	ALOH (n=20)	ALOH & PM (n=20)	p
Min-Max	2.02-3.35	78.55-104.94	36.06-62.48	
Mean ± SD	2.86 ± 0.50	91.59 ± 9.18	42.21 ± 7.30	<0.001*
Median	2.93	88.77	41.12	
Sig. bet. grps.	p_1<0.001*, p_2<0.001*, p_3<0.001*			

Note: p: p value for Kruskal Wallis test, p_1: p value for comparing between control group and ALOH group, p_2: p value for comparing between control group and ALOH & PM group, p_3: p value for comparing between ALOH group and ALOH & PM group, *: Statistically significant at p ≤ 0.05

Surface area of the testes tubules: Illustrated the mean level of surface area of the testes tubules in control group was 124.60 ± 6.42. The mean level of group II (ALOH) was 66.77 ± 9.38, thus significantly decreased as compared to the control group. The mean level of group III (ALOH & PM) was significantly increased as compared to group II, being 97.91 ± 4.05. There was a significant difference between the three studied groups regarding surface area of the testes tubules (p<0.001) (Table 2).

Table 2. Comparison between the three studied groups according to the surface area of the testes tubules.

The surface area of the testes tubules	Control (n=20)	ALOH (N=20)	ALOH & PM (n=20)	p
Min-Max	117.0-134.0	57.54-78.35	93.85-104.88	
Mean ± SD	124.60 ± 6.42	66.77 ± 9.38	97.91±4.05	
Median	123.35	63.33	97.36	<0.001*
Sig. bet. grps.	p_1<0.001*, p_2<0.001*, p_3<0.001*			

Note: p: p value for ANOVA test, p_1: p value for comparing between control group and ALOH group, p_2: p value for comparing between control group and ALOH & PM group, p_3: p value for comparing between ALOH group and ALOH & PM group, *: Statistically significant at p ≤ 0.05

Count of sertoli cell: Illustrated the mean level of number of sertoli cells in the seminiferous tubules in control group, group II and group III which were 1.20 ± 1.15, 101.0 ± 23.60 and 22.50 ± 8.51 respectively. The mean level in group II (ALOH) was significantly increased as compared to the control group. The mean level in group III (ALOH & PM) was significantly decreased as compared to group II. There was a significant difference between the three studied groups regarding number of Sertoli cell in the seminiferous tubules (p<0.001) (Table 3).

Table 3. Comparison between the three studied groups according to the number of Sertoli cells in the seminiferous tubules.

The number of sertoli cells in the seminiferous tubules	Control (n=20)	ALOH (n=20)	ALOH & PM (n=20)	p
Min-Max	0.0-3.0	80.0-150.0	10.0-30.0	
Mean ± SD	1.20 ± 1.15	101.0 ± 23.60	22.50 ± 8.51	
Median	1	90	25	<0.001*
Sig. bet. grps.	p_1<0.001*, p_2<0.001*, p_3<0.001*			

Note: p: p value for Kruskal Wallis test, p_1: p value for comparing between control group and ALOH group, p_2: p value for comparing between control group and ALOH & PM group, p_3: p value for comparing between ALOH group and ALOH & PM group, *: Statistically significant at p ≤ 0.05

Integral optical density of The CK 18 immunostaining: The mean level in control group was 38.21 ± 8.56, in group II (ALOH) was 114.34-12.21 and in group III (ALOH & PM) 72.11 ± 11.05. There was a significant increase in group II as compared to the control group, a significant decrease in group III as compared to the group II. There was a significant difference between the three studied groups

regarding integral optical density of the CK 18 immunostaining ($p<0.001$) (Figure 3).

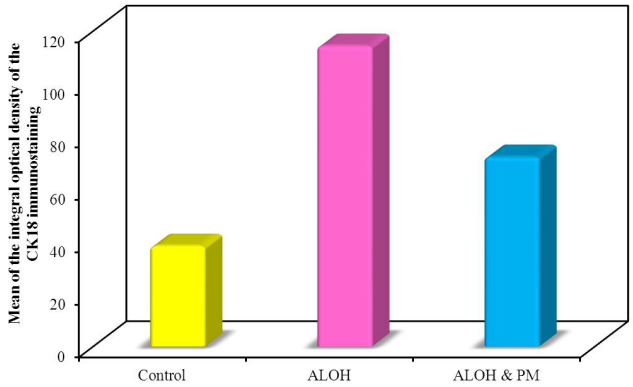

Figure 3. Illustrated the mean level of integral optical density of the CK 18 immunostaining.

Discussion

The exposure to metals is inevitable by people because of their universality in nature, contaminated air, water, food and soil, long-term persistence in the environment and wide use in industry. Metals have been shown to influence spermatogenesis in rodents and people, which can lead to abnormal sperm morphology, low sperm count and poor semen quality [13]. AL is one of the most widely distributed metals in nature and is widely utilized as a part of day by day life that gives easy exposure to individuals. It is poorly absorbed and accumulated in tissues resulting in their dysfunction and toxicity. The testes are known to be very sensitive and vulnerable more than many other tissues to stress produced by many environmental or occupational hazards including heavy metals, the issue that might lead to infertility and congenital defects [14].

In the present study, the gross appearance of rat testes by stereomicroscopic examination revealed destructive seminiferous tubules, presence of hemorrhages between the tubules, thickening of tunica albuginea and small surface area of the testes tubules which were statistically significant in comparison to the control group ($p<0.001$). In group III (administrated ALOH & PM), the rats' testes showed few destructive seminiferous tubules, others were homogenous with reduction of hemorrhages between the tubules. Also, a marked recovery of thickness of tunica albuginea and surface area of the testes tubules were statistically significant decreased in comparison to ALOH group ($p<0.001$). This finding revealed the toxic effect of AL metal on the rats testes tubules and the restoration of the histomorphological findings caused by administration of promgeranat molases.

The light microscopic examination showed that the semineferous tubules had lost its characteristic architecture compared to the control. Some seminiferous tubules attained different shapes, had disturbed basal lamina and disorganized germinal epithelium. In some seminiferous tubules, the germinal epithelium was thick containing hyperprolifirating cells with hyperchromatic nuclei; these cells were non-functioning with dead spermatids. They were separated from the basal lamina and had multiple intercellular spaces due to germ cells desquamation

Similarly, other studies on rats' testes after rat's exposure to AL revealed that the germinal epithelium was thin with decreased number of spermatogenic cells; consequently the lumen of the tubules appeared wide with few or no observed sperms (maturation arrest). In the interstitial tissue, widening of the tissue, dilatation with engorgement of the interstitial blood vessels and interstitial vacuolation were noticed [14,15]. It may be to suggest that these changes were due to the effect of AL accumulation in male rat testes.

In line with our finding, Afeefy et al., reported that the rats' exposed to ALOH, a testicular damage lead to spermatogenic arrest [13]. The seminferous tubules were distorted with undifferentiated germinal epithelium. The spermatogenic cells were undifferentiated and separated from the basement membrane. The spermatogenic cells decreased in number to few layers, consequently the wide lumen of the tubules and contained desquamated epithelial cells and no sperms were observed. Thickened blood vessels and hyaline materials were appeared in most tubules. This impairment caused by prolonged accumulation of ALOH in the rat testes.

A histological perturbation, in rats testes after ALCL treatment, revealed severe damage within the seminiferous tubules and vacular degeneration on the spermatogenic and sertoli cells cytoplasm, as well as marked distorted seminiferous tubules with loss of normal distribution of epithelial linning and vacuolar cytoplasm after rats' exposure to various concentrations of ALCL [16,17]. The presence of ALCL in drinking water has a suppressive effect on the fertility of male rats. These changes were dose dependent to suggest that these changes were due to the effect of AL accumulation in male rat testes [1]. In addition, a significant decrease in ejaculate volume, total sperm output, sperm motility, normal and live sperm and an increase in dead and abnormal sperm were observed in rabbits orally administered ALC, that AL ingestion in excessive amounts led to damage of testicular tissues of both human and animals [15]. Also, in male mice treated intraperitoneally with aluminum nitrate demonstrated necrosis of spermatocytes with a significant decrease in fertility [5]. These results revealed the high toxicity of AL metal preciptated and accumulated in animal tissue. In addition, the nitric oxide produced by AL was responsible for allowing AL to enter the tight junctions that form the inter-Sertoli (so called blood-testis) barrier and accumulated in the testis coused damages germ cells, Sertoli cell and the seminiferous epithelium with a decrease in its differentiation, thus altering normal spermatogenesis and sperm production, as well as, thining of germinal epithelium of the seminiferous tubules, absence of spermatids and sperm numbers in the lumen [18]. So, altered sperm morphology causing decreased sperm motility and viability. An increase in germ cell apoptosis and subsequent hypospermatogenesis are caused by AL induced oxidative stress and also it leads to a fall in intercellular ATP level which decreases sperm motility [5].

The oxidative stress could be protected by medicine plant rich in polyphenol compound such as pomegranate molasses (PM) [6]. Pomegranate fruit, peel and juice have a marked antioxidant capacity because it has a high content of polyphenols. The soluble polyphenol content varies within the limits of 0.2%-1.0%, depending on variety, and includes mainly anthocyanins, catechins, condensed tannins, ellagitannins, gallic and ellagic acids [19,20]. It has several pharmacological activities because of its marked antioxidant effect, it has been suggested as anticarcinogenic, chemo-preventive,

chemotherapeutic, antimicrobial, anti-inflammatory and antiatherosclerotic [21].

In the present study, stereomicroscopic examination of testes of rats administrated ALOH & PM showed few destructive seminiferous tubules; others were homogenous with reduction of hemorrhages between the tubules, there was a significant decrease of thickness tunica albuginea ($p<0.001$) and a significant increase of surface area of the testes tubules ($p<0.001$) in comparison to the ALOH administrated group.

The light microscopic examination of rat testes administrated ALOH & PM (group III) showed marked improvement in the histological structure than those of ALOH treated rats. There was a marked restoration of the normal shape of the seminiferous tubules with intact basal laminae. The germinal epithelium became well stratified and well organized with recovering of the differentiated spermatogenic cells with abundant free spermatozoa in the lumen. Marked improvement of interstitial tissue in between the tubules was also observed. Simillary, the study of pomegranate juice consumption provided an increase in epididymal sperm concentration, sperm motility, spermatogenic cell density and diameter of seminiferous tubules and germinal cell layer thickness, and it decreased abnormal sperm rate when compared to the control group. This finding represented that the pomegranate molasses may have a protective role against the toxic effect of ALOH [19]. This result was simillary deduced that pomegranate extract can be useful for the treatment of the deleterious effect of lead acetate administration on sperm production in rats as pomegranate prevented lead induced spermatogenic disruption in rats and its antioxidant activity could explain its capacity to reverse the damage produced by lead on spermatogenesis [22]. Pomegranate juice augments the antioxidants defense mechanism against carbon tetrachloride-induced reproductive toxicity and provides evidence that it may have a therapeutic role in free radical mediated diseases [23].

Additionally, the present study used the immunohistochemical technique to evaluate the defect of the signal marker expression in damaged testes CK 18 protein is consistently expressed in immature Sertoli cell of prepubertal seminiferous tubules, but is completely absent in normal, mature seminiferous tubules. The normal maturation of the sertoli cell is characterized by the absence of cytokeratin filaments, making it different from the other true epithelia. Expression of CK in adult seminiferous epithelium is regarded as a sign of either maintaining or regaining undifferentiated immature features [24]. Testicular disorders resulting in infertility are associated with the presence of CK18 in sertoli cells. This may be a primary event indicating a failure of maturation of sertoli cells or a reversion of sertoli cell to a less differentiated state because of the absence of normal spermatogenic progression [24]. In the present study, immunohistochemical detection of CK 18 in control group was a very weak expression, a strong CK expression was shown in ALOH administrated group II and a moderate expression was found in ALOH & PM adminstrated groupIII. This result revealed that the CK 18 is absent in the mature testes and its expression was marked to the injuried Sertoli cells. The expression of the CK 18 distributed as strong positive brown granules in the sertoli cell which hyperproliferated and migrated from the periphery of the seminiferous tubules to the lumen in the ALOH administrated group. Whereas, the CK18 was expressed as moderate brown color in the injuried sertoli cell which was localized at the periphery of the seminiferous tubules in ALOH & PM administrated group. So the present finding revealed that Pomegranate molasses can be useful and has augmented of the antioxidant defence aganist effect of ALOH administration on Sertoli cell activity in rats and prevented spermatogenic disruption, the antioxidant activity of pomegranate could explaine its capacity to reverse the damage produced by ALOH on Sertoli cell function and differentiation of spermatogenesis.

Conclusion

The present histopathological resuts noticed the hyperproliferating spermatogenic cells appeared as cells with hyperchromatic nuclei fill the lumen, while by immunohistochemical staining, these cells were identified to be immature hyperproliferating sertoli cell (which stained by CK18), that hyperproliferated as a compensatory mechanism against the destructive effect of ALOH but without a significant function because they were immature and there were no or few sperms inspite of its hyperproliferation in the sminefrous tubules. Moreover, the image analysis used to confirm the present result. There was a significant increase in the number of CK 18-positive sertoli cell in ALOH group compared to the control group and a significant decrease in ALOH & PM administrated group compared to the ALOH group ($p<0.001$). The integrated optical density of the CK18 expression revealed a significant increase of the CK18 immunostaining in the ALOH group compared to the control group and a significant decrease in ALOH & PM administrated group compared to the ALOH group ($p<0.001$). These results revealed that the immunohistochemical detection of cytokeratin 18 in Sertoli cell may be provided a sensitive marker for immature or damaged testes. It has been known that Sertoli cell number in the testis establishes the upper limit of sperm production because each Sertoli cell supports a relatively fixed number of germ cells. Therefore, the regulation of Sertoli cell proliferation induced by the ALOH administration was impaired nutrition and structural support for germ cell. Whereas the reduction of the positive Sertoli cell number and expression of the CK18 in ALOH & PM group revealed the best role of the PM as protective agent and its safty reagent for intake.

Therefore, the present findings revealed the ALOH caused damage in rat testes due its accumulation in tissue, this degeneration of the testes lead to increase in the collagen fiber and increased the expression of the CK18 protein as well as the randomly distribution of the Sertoli cells. These changes revealed the toxic effect AL on the rate testes. The PM intake with ALOH revealed recovery of the testes architecture, as well as, reduced the collagen fiber content and the CK18 expression in the Setroli cells. The PM may have a protective role in rat testes. The immunohistochemical detection of CK 18 in Sertoli cells may be provided as a sensitive marker for immature or damaged testes.

Overviewing the results of the present study, it could be concluded that; the ALOH caused damage in rat testes due its accumulation in the tissue, this degeneration of the testes lead to increase the expression of the CK18 protein as well as the randomly distribution of the Sertoli cells. The PM intake with ALOH revealed recovery of the testes architecture, as well as, reduced the CK18 expression in the Setroli cells. The PM may have a protective role in rat testes. The immunohistochemical detection of CK 18 expression in Sertoli cells

may be provided as a sensitive marker for immature or damaged testes.

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