

Comparison of Ferulic Acid and Sildenafil, Therapeutic Role against Testicular Injury Induced by Cadmium Chloride in Adult Male Albino Rat. (A study using Light & Atomic-Force Microscopes "L.M & AFM" and Gene expression by Real-Time PCR)

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Abstract: *Cadmium chloride considered one of the toxic heavy metals for both animals and human and can induce damage in animal organs testis, kidney and liver. Testis is the main organ to be affected by lowering fertility. This was clarified in this study by histological, biochemical and laboratory findings (semen and hormonal analysis). Ferulic acid (FA) is a common polyphenolic compound present in vegetables, as, eggplants and is an antioxidant preventing lipid oxidation in food and consequently prevents free-radical-induced diseases. Sildenafil citrate (causes dilation of peripheral veins and arteries sused for the treatment of erectile dysfunction).*

Results showed that cadmium chloride was found to induce increased testicular tissue roughness using the "AFM" and induced sever damage of seminiferous tubules that might be up to necrosis as seen by using the "LM". This was associated with increased gene expression of

B-Cell/ Lymphoma 2 (Bcl 2) , cyclooxygenase-2 (COX-2) and Metallothionein 1(Mt1) , a significant decrease in inhibin- B level which was associated with insignificant increase in testosterone and significant increase in T3 & T4 levels ,while decreasing count and viability of sperms in semen analysis.

Whereas, treatment with ferulic acid as well as sildenafil significantly increased the level of inhibin-B. Treatment with only sildenafil significantly increase the count and viability of sperms, while treatment with ferulic acid only significantly increased the viability of sperms but decreased their count.

Aim of the Study: *This work is designed to find out the effect of cadmium chloride on histological, biochemical, and hormonal parameters of the reproductive organ "testes" of adult male rats and showing the protective effect of Ferulic acid versus sildenafil on testicular damage.*

Keywords: *Ferulic acid, sildenafil, cadmium chloride, testicular injury, inhibin-B, metallothionein.*

1. INTRODUCTION

Cadmium is a toxic heavy metal of environmental concern which after exposure leads to adverse effects, [1]. Cadmium have a cumulative toxin, due to its long biological half- life so the residual metal induce direct toxic effects,[2].Cadmium long residence as a result of a metal-binding protein metallothionein (MT), [3].The testes of the rat are sensitive to tumor genesis induced by cadmium when given with high dose, leading to necrosis and hemorrhagic testes, [2]. Ferulic acid (FA) is a common polyphenolic compound present in vegetables, as, eggplants, artichokes (~90% of total polyphenols) [4]. Ferulic acid is one of compounds in rice bran pitch, which is obtained when rice oil is produced, [5]. This antioxidant compound prevent lipid oxidation in food and prevent free-radical-induced diseases such as aging caused by oxidative tissue degeneration, atherosclerosis and cancer, [6]. Sildenafil citrate, considered a specific inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type-5 (PDE-5), causes dilation of the peripheral veins and arteries due to enhanced nitric oxide (NO) synthesis [7], so used for treatment of erectile dysfunction. Several studies investigated the lipid peroxidation inhibition by cGMP analogues and cyclic adenosine

monophosphate [8]. PDE-5 inhibitors have a protective effect in ischemia/reperfusion injury to the testes, kidney, brain, spinal cord, ileum, and myocardium, [9]. Sertoli cells produce inhibin, which is a protein hormone suppress FSH secretion by the anterior pituitary gland through a negative feedback Loop, [10]. There are two forms of inhibin, A and B. Both consist of a subunit which is linked via disulphide bridges to one of two different b- subunits (bA or bB). Inhibin-B is the circulating form in all species. With the recent development of specific two-site immunoassays, it is possible to analyze the different forms of inhibin and to distinguish them from the inactive free- inhibin subunits [11].

2. MATERIALS AND METHODS

2.1. Animals

Twenty four adult male albino rats (Sprague Dawley strain; 150±20 gm of mean body weight) was purchased from the Laboratory Animal Unit of Nile Center of Experimental Research Mansoura Egypt were used in this study. The rats were allowed for 2 weeks acclimatization period. Thereafter, they were randomly divided into four groups of 10 rats each. They received water and chow ad libitum throughout the period of the experiment.

2.2. Cadmium: was purchased from Thomas Baker Chemical Industries, CAS. No. 35858-65-2

2.3. Ferulic Acid: was purchased from sigma Aldrich cat.:12,870-8, 99%

2.4. Sildenafil: as sildenafil citrate was purchased from Pfizer Inc. (Pfizer, Egypt).

2.5. Preparation of Sildenafil

According to, [12]. The average dose for human (62.5 mg/day) was converted to equivalent dose for rat. The rat dose was calculated as 5.625 mg/kg body weight. Thus, a rat weighing 250 gm given a dose of 1.5 mg suspended in 1.5 ml distilled water orally. Each 100mg tablet of sildenafil citrate dissolved in 100 cubic cm of D.W., so each 1 cubic cm contained 1mg of the drug. So every 250 gm of the rat received 1.5mg of the drug = 1.5 cubic cm of the drug solution, [13].

2.6. Experimental Protocol

Twenty four adult male rats were allocated to one of four groups of six rats of each and subjected to the following treatments: **Control (G1)** six rats served as control and received 0.5ml of saline by intra-peritoneal injection (i.p.) route as a single dose. **Cadmium (G2)** six rats received i.p. injection of cadmium chloride dissolved in normal saline in a single dose of 2 mg/ kg bodyweight [14]. **Ferulic acid (G3)** six rats received cadmium and **ferulic acid**. The doses of ferulic acid selected based on previously reported protective and antioxidant properties of this compound in rats (40 mg/kg body weight) for 2 weeks. **Sildenafil (G4)** rats received cadmium and sildenafil 1mg/kg dissolved in 0.9% NaCl injected (i.p.) for 2 weeks. At the end of the experiment, the rats of each group were fasted overnight, and sacrificed under slight ether anesthesia. Blood was collected by cardiac puncture. Serum were separated by centrifugation at 860 xg for 20min. and determination of testosterone hormone and T3& T4.

After the collection of blood, the testis were rapidly excised from each animal, and washed with 0.9 % NaCl solution, part of it was minced and homogenized for determination of

Inhibin-B, Another part of the testis was washed in 0.9% saline, minced and homogenized, for determination of Bcl2, COX-2 and metallothionein enzyme using quantitative real time PCR (RT-PCR, ARKTIK, thermal cycler, USA).

Fresh specimens were taken from the testis of each animal and dissected into pieces; and immediately fixed in 10% formal saline and paraffin – embedded. The specimens were prepared for histological study; for light microscope using Haematoxylin and Eosin stain (H&E) and for atomic force microscopic examination [14].

2.7. Gene Expression by Real-Time PCR

Evaluation of Bcl2, COX-2 and Mt1 expressions were performed using Real-Time thermal cycler (CFX96, Bio Rad, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as an internal control and for normalization. Briefly, Extraction of total RNA from tissue in different groups was done by RNeasy Mini kit (QIAGEN GmbH, Germany). The concentration of RNA was measured by spectrophotometer (Nanodrop 2000, Thermo Scientific, USA) and 1 µg of total RNA was converted to cDNA using RT2 First Strand Kit (QIAGEN Science, Maryland, USA). Then, 3 µl (about 30 nM) of the cDNA was amplified using 10 pmol of each primer pair, 10 µl of 2× RT2 SYBR

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Green Master Mix (QIAGEN Science, Maryland, USA), and nuclease-free water to a total volume of 20 µl. The cycling parameters of the PCR amplification were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of amplification (denaturation at 94 °C for 20 seconds, annealing 58 °C for 30 seconds and extension at 60 °C for 30 seconds). For each sample, the procedure was carried out in triplicate. The primer design was performed on line at NCBI site and the primer sequences for each gene were showed in Table (1). A mathematical model introduced by [15] was used for the relative quantification of target genes. In this study, gene expression was expressed relative to that of control group (G1).

Table1. List of Rat Gene-Specific Primers in Real-Time PCR

Gene	sense	Anti-Sense	
glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	TGCCACTAGAAGACTGTGG	GGATGCAGGGATGATGTTCT	NM_017008.4
Metallothionein 1(Mt1)	CCTCCTGCAAGAAGAGCTGC	CACTTCAGGCACAGCACGT	NM_138826.4
cyclooxygenase-(COX-2)	TCCTCCTTGAACACGGACTT	CTGCTTGACAGCGATTGGA	NM_017232.3
B-cell B-Cell/ Lymphoma 2 (BCL 2)	GTACCTGAACCGGCATCTG	ATCAAACAGAGGTGCGCATGC	NM_016993.1

2.8. Atomic Force Microscope (AFM) Image and Measuring Roughness

AFM imaging was conducted in Nanotechnology center, Mansoura University, Mansoura, Egypt. Roughness of the testicular tissue was measured according to the method of [16]. The AFM imaging was conducted using contact mode using uncoated sharpend micro-levers and sharp-end tip with 10nm radius NANOSUR FLEX AFM (NANOSURF AG, SWITZERLAND). For an AFM probe, silicon cantilever with spring constant of 0.2N/m.scans were performed in air. The tip cantilever length was 225µm, width was 38µm, resonance frequency was 190 kHz and force constant was 48 N/m. Image acquisition was carried out using iNano SPM software. Sampling points were set at 256.

2.9. Statistical Analysis

All values were presented as mean ±SEM. Differences were considered to be significant at p<0.05. One-way analysis of variance (ANOVA) and post-hoc test were used to determine differences between groups. The SPSS/PC program (version 17; SPSS, Chicago, Illinois, USA) was used for statistical analysis according to [14].

3. RESULTS

The results of biochemical parameters demonstrated that the rats treated with cadmium caused significant (P≤0.05) testicular damage as evidenced by hormonal analysis, testicular inhibin-B (pg/ml) and semen analysis (table 2).

Table2. T3,T4, Inhibin-B, and semen analysis in control group and in different treated groups

Group/Parameters	Control	cadmium	Sildenafil	Ferulic acid
T3	148.33±4.07a	247.83±7.79b	214.50±1.80c	123.70±11.51d
T4	8.68±0.29a	14.66±0.44b	11.29±0.36c	7.85±0.21a
Inhibin-B	0.33±0.01a	0.18±0.03b	0.52±0.03c	0.34±0.01a
Semen analysis				
Count/million/ml	82.17±1.70a	49.27±0.98b	67.25±0.82c	29.10±0.95d
Viability	Viability	53.15±1.41b	67.47±0.54c	72.61±1.64d

Each value represent the mean ± SD, values superscripts with different letters (a-d) were significantly different at p≤0.05. testosterone level was insignificant so it was not mentioned.

In cadmium to treated group (G2), there was significant (P≤0.05) increase in T3 and T4 and insignificant increase in testosterone, while significantly (P≤0.05) decreased inhibin- B level (pg/ml) when compared with the control group (G1). In ferulic acid treated group (G3), there was significant (P≤0.05) decrease in the activity of serum T3 and T4, significant (P≤0.05) increase in inhibin-B, and sperm viability but decreased sperm count. However, in sildenafil treated group (G4), there was significant (P≤0.05) decrease in the activity of serum T3 and T4, where significant (P≤0.05) increase in inhibin-B, sperm count and viability when compared with the control group (G1). Moreover, the count of the sperm in sildenafil group was much more than ferulic acid group where the viability percentage was higher in ferulic group more than sildenafil group (table 1)

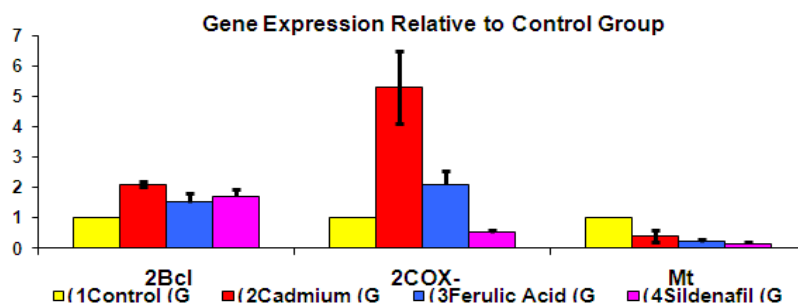
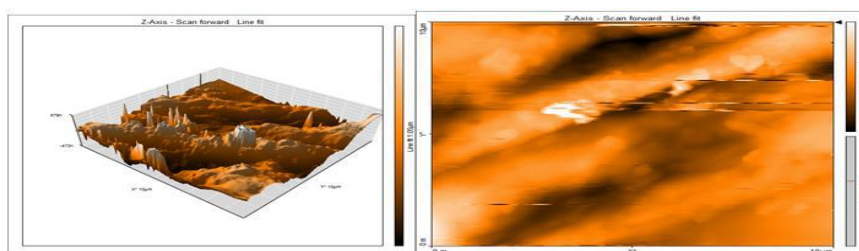


Fig1. The fold expression changes in three genes for different groups which normalized by GAPDH gene and relative to that of control group (G1).

Gene Expression The fold changes in the gene expression for Bcl2, COX-2 and Mt were detected by Real-Time PCR and the concentration for each practical test was normalized to its GAPDH reference gene according to the equation [16]. Each test also was done in triplicate for the all samples in each group. The mean values and standard errors were calculated and showed in **Fig.1**. Bcl2 gene was expressed more in the positive group of cadmium (2.1 ± 0.09) where in other treated groups G3 and G4 was less (1.5 ± 0.3 and 1.7 ± 0.23), respectively.

COX-2 expression was also increased highly in the positive group (G2) while decreased in other groups G3 and G4 than positive cadmium (5.3 ± 1.2 , 2.1 ± 0.43 and 0.51 ± 0.77), respectively. The expression of Mt gene was less than normal in all groups but the treated groups were observed more less (0.42 ± 0.2 , 0.2 ± 0.08 and 0.14 ± 0.44).



Name	Value
Area	100.4pm ²
Sa	141.91nm
Sq	176.01nm
Sy	2290.7nm
Sp	1.498µm
Sv	-792.72nm
Sm	-9.6912fm

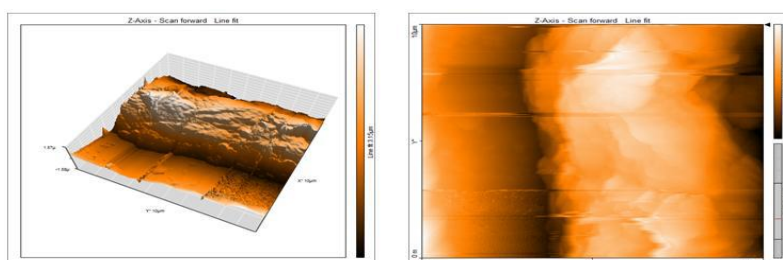


Fig2.A

Name	Value
Area	100.4pm ²
Sa	597.12nm
Sq	695.44nm
Sy	3.2429µm
Sp	1578.4nm
Sv	-1664.4nm
Sm	-16.859fm

Fig2.B

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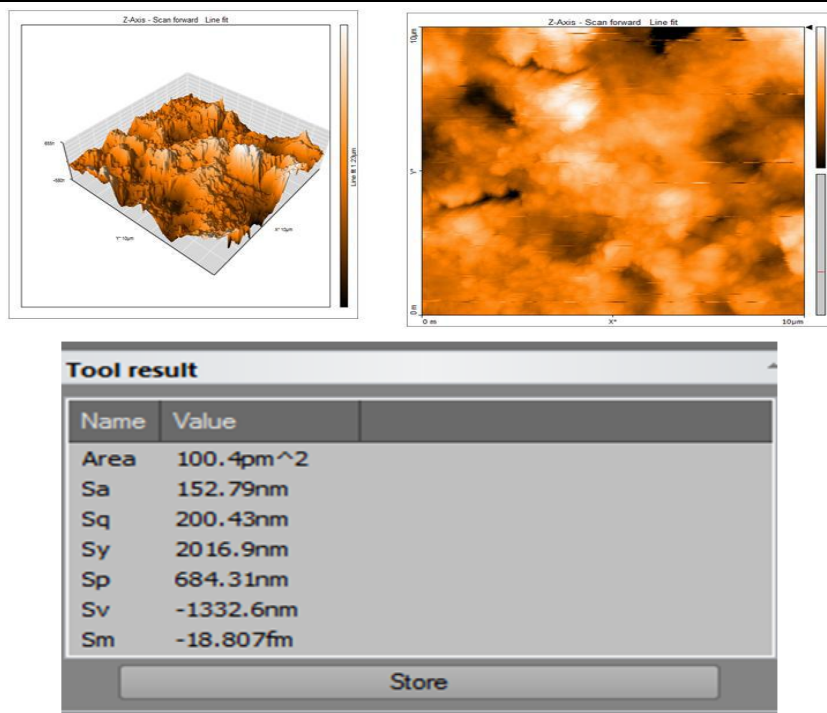


Fig2.C

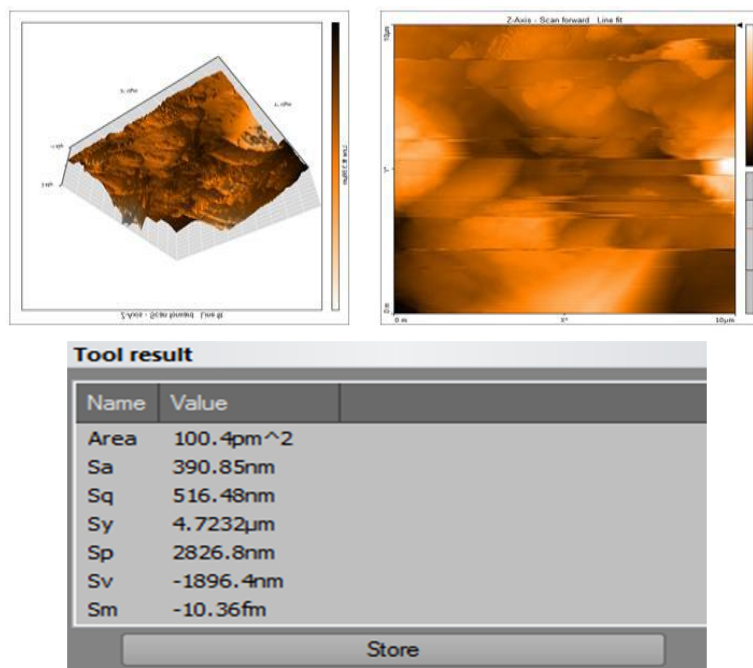


Fig2.D

Fig2. AFM image of testis in control (a) and cadmium(b) treated rats , in sildenafil (c) and ferulic acid(d) treated rats .A significant increase in roughness referred to sq(176.01nm)(a) in cadmium (b) treated rats (695.44nm), where in sildenafil(c) treated rats roughness referred to sqwas (200.43nm). In rats administered with ferulic acid roughness referred to sq was (516.48nm).This means that sildenafil reduced the roughness of testicular tissue towards normal level.

- Sathe roughness average
- Sm.....the mean value
- Sqthe root mean square
- Svthe valley depth
- Spthe peak high
- Sy.....the peak-the valley high

Histological study of the testis

Light microscopic examination using H&E stain: Sections of the testicular tissue from control group (G1) showed normal structure of the testis. The seminiferous tubules appeared rounded or oval with regular basement membrane and lined with stratified germinal epithelium including all types of progenitor cells (fig.3). Sperms were seen in the lumina of the tubules. The narrow interstitial spaces contained clusters of lightly stained interstitial cells of Leydig and blood capillaries in-between (fig.3)

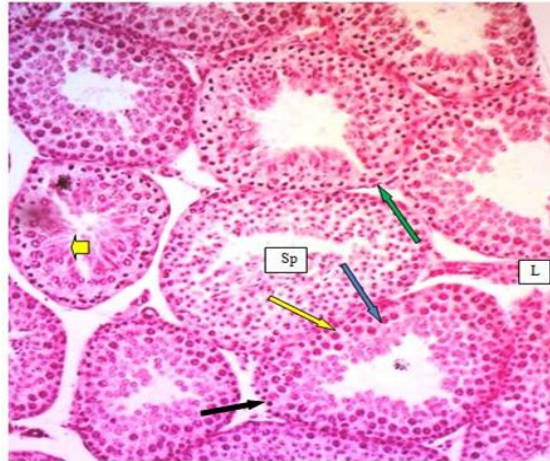


Fig3. A photomicrograph of a section in a control adult albino rat's testis showing seminiferous tubules. Spermatogenic cells resting on regular basement membrane; spermatogonia (black arrow), primary spermatocytes (yellow arrow), secondary spermatocytes (blue arrows), spermatids (sp) and sperms (arrow head), are seen in their lumina. Narrow interstitial spaces contain lightly stained Leydig cells (L). Each tubule is ensheathed by a single layer of myoid cells (green arrow). [H&E X 400]

Sections obtained from the testes of cadmium-treated group (G2) revealed seminiferous tubules with severe damage and might be necrosis. Some seminiferous tubules appeared shrunken and had different shapes with irregular outlines, wide lumina and disorganized epithelium. The interstitial spaces are wide and had Leydig cells with darkly stained nuclei (fig. 4A). Other seminiferous tubules showed sloughing of germ cell layers with marked vacuolations and the lumina of these tubules were filled with degenerated germ cells (fig. 4B). Marked necrosis of certain areas of the testicular tissue in the form of peri-vascular hemorrhages and inflammatory cell infiltration (Fig. 5).

In cadmium and ferulic acid – treated group (G3), wide areas of testicular tissue were more or less similar to the control group (Figs.6 A, B). In cadmium and sildenafil - treated group (G4) most of seminiferous tubules nearly retained their normal structure. They revealed regular rounded contour and were lined by stratified germinal epithelium showing several types of spermatogenic cells. Most lumina contained aggregations of sperms. The interstitial spaces contained clusters of Leydig cells but still with darkly stained nuclei and some blood capillaries appeared congested (Figs. 6 C).

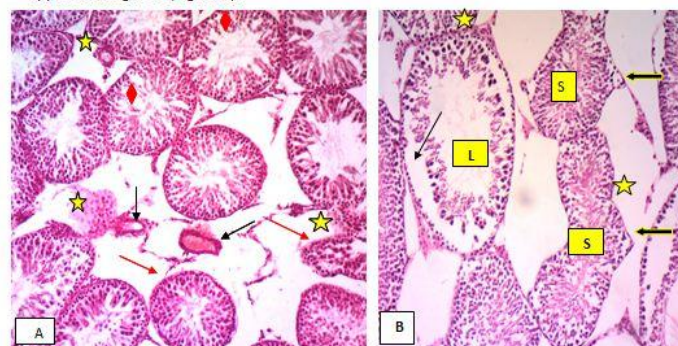


Fig4. photomicrographs A & B of a section in albino rat's testis of cadmium treated group, A; showing multiple shrunken tubules (red arrows) and the tubules have different shapes with apparent disorganization of germinal epithelium, and marked vacuolations (◆). The inter-tubular spaces are wide with congested blood capillaries (black arrows) and interstitial cells with darkly stained nuclei

B; showing sloughing of germ cell layers (arrow), some tubules resting on an irregular basement membrane (thick arrows). Some tubules have wide lumina (L). Few tubules appear with aggregation of sperms (s). The wide interstitial spaces contain Leydig cells (◆) with darkly stained nuclei [H&E X200]

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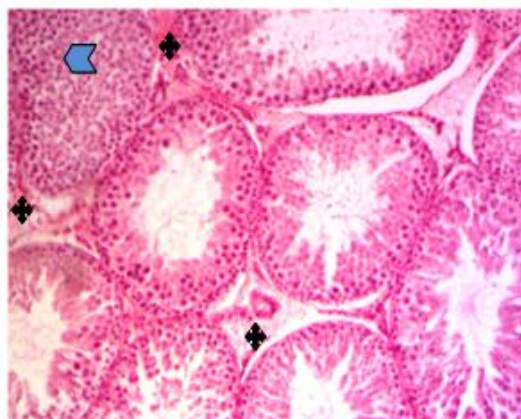


Fig5. Testis of cadmium treated rats showing necrosis of seminiferous tubules (arrow), congested blood capillaries and peri-vascular hemorrhages (◆) and inflammatory cells infiltration (arrowhead), [H&E, X200]

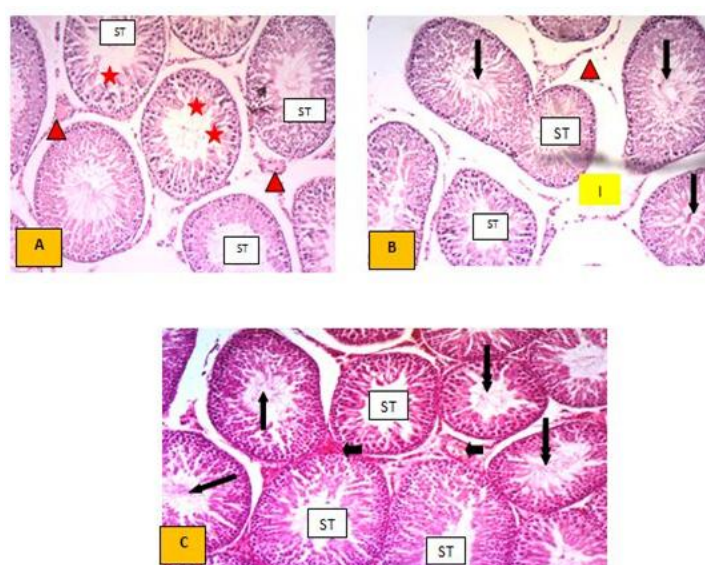


Fig6. Photomicrographs of the testis of treated groups with Ferulic Acid (A& B) and with Sildenafil (C). A; showing some of the seminiferous tubules (ST) still have disorganized epithelial lining, vacuoles (stars), and lumina contain degenerated germ cells. The interstitial spaces contain dilated blood capillaries and clusters of Leydig cells with darkly stained nuclei (arrow heads). B; showed seminiferous tubules (ST) lined with several types of spermatogenic epithelium and lumina contained aggregations of sperms (arrows). Interstitial spaces (I) are still wide

C; showed the testis of Sildenafil treated group. In which most of the seminiferous tubules (ST) are more or less normal with nearly regular & rounded contour and are lined by stratified germinal epithelium. Most lumina contain aggregations of sperms (arrows). The interstitial spaces contain clusters of Leydig cells and dilated blood capillaries (arrow head). [H&E X 200]

4. DISCUSSION

Cadmium is considered the most pollutants for environment in the biosphere, [17]. The risk factors for occupational exposure by uses of cadmium in glass, plastics, fertilizers, and batteries. The major sources for non occupational cadmium exposure were, air pollution, tobacco smoking, and consumption of contaminated drinking, [18]. Several dysfunction and injury of body organs, as the testes, were reported due to toxicity with cadmium in humans and animals, [19]. Several studies demonstrated that toxicity with cadmium causing testicular damage, and compromised testicular function, [20].

Light microscopic study of cadmium treated group of this study showed some seminiferous tubules had morphological changes in the form of multiple disorganization, vacuolations and marked sloughing of the epithelial lining. Severe damage and necrosis of the testicular interstitium. Some tubules appeared shrunken with decreased epithelial thickness and wide lumina. This led to wider interstitial spaces. Other seminiferous tubules showed disintegrated and degenerated germ cells. This

might be attributed to impairment of the function of Sertoli cells in engulfing the degenerated cells. Similar findings were reported by [21], [22] and [23]. These authors reported that Sertoli cells are recognized to play an important role in spermatogenesis, i.e. normal sperm requires normal Sertoli cell. In addition, the separation of spermatids and spermatocytes from Sertoli cells would interfere with the transfer of nutrients from Sertoli cells. This led to inhibition of spermatogenesis and led to death and disintegration of the germ cells.

In this study it was also found that in cadmium-treated group the spermatogenic cells and also the interstitial Leydig cells appeared with darkly stained nuclei (pyknosis). [24] stated that cadmium produced an extensive germ cells apoptosis in Sprague-Dawley rats. Apoptosis is a physiological process that contributes to keeping the cell number in testicular tissue and helps to remove damaged cells, but excessive apoptosis could cause destruction of male reproductive function [25].

In the ferulic acid and Sildenafil-treated groups in the current study the normal histological structure of the testes was more or less preserved. Testicular architecture was apparently normal if compared with the control group. However, few seminiferous tubules showed disorganization and focal vacuolations of the germinal epithelium.

In accordance with this study, several authors had reported that use of substances having antioxidant activities, such as vitamin C, vitamin E, Zn, selenium and melatonin might be very useful in protecting the tissues against cadmium toxicity. They also demonstrated that these substances reduced and/or prevented both the oxidative stress and the subsequent testicular damage [26, 27].

Sildenafil used as protection against ischemia/reperfusion-induced tissue injury in other organs including brain, colon, and liver, [27-28 and 29] As well as Sildenafil reduces ischemia/reperfusion testicular injury after torsion/de-torsion in rats [30]. Ferulic acid considered antioxidant, anti-carcinogenic, anti-microbial, anti-hyperlipidemic anti-hypertensive, anti-inflammatory, anti-diabetic, neuro-protective, hepato-protective and radio-protective properties. It is also used in the treatment of the age-related diseases. [31, 32]. It has been shown that inhibin-B is the only physiologically important hormone in rats and in men, [33]. In the present study, administration of cadmium caused a decrease in inhibin-B compared with control group. This was associated with insignificant increase in testosterone and significant increase in T3&T4 levels. This study showed that Testosterone increased T3/T4 ratios.

These results might be due to direct inhibitory effect of cadmium on production of inhibin-B from Sertoli cell. This is in agreement with results of [34].

However, it was found in the present study that toxic effect caused by cadmium was ameliorated with ferulic acid and sildenafil, where the tissue inhibin-B level was restored and elevated while T3&T4 level was decreased. But there was more pronounced effect with sildenafil than with ferulic acid. It could be said that inhibin correlated negatively with T3& T4 and consequently with testosterone. This was in accordance with [35]

who reported that increases in the levels of inhibin in interstitial fluid "IF" of seminiferous tubules and of FSH and LH in serum while testosterone levels in IF and serum fell to undetectable levels.

These results confirmed by atomic force microscope where degree of roughness of testicular tissue was high in cadmium-treated group and decreased in ferulic acid-treated group (516.48nm) but more lower in sildenafil-treated group (200 nm).

Our results demonstrated that rats injected with cadmium showed increased gene expression of Bcl2, COX2, and MT. Increase expression of COX2 gene most probably due to initiation of inflammatory response. MT gene expression increased as a defense mechanism against cadmium toxicity. Increased Bcl2 gene expression might be due to germ cell apoptosis induced by cadmium. This finding was in agreement with [36, 37 and 38]. In the present study in groups 3&4 treated with ferulic acid and sildenafil, gene expression of Bcl2, COX2, and MT was lowered. This means that using these drugs "sildenafil and ferulic acid" prevent the inflammatory response and preserve the integrity of germ cells.

5. CONCLUSION

In conclusion, the present study suggested that treatment with sildenafil and ferulic acid might induce curative effect against testicular injury and protected the testes exposed to heavy metals "cadmium", but sildenafil effect was better than ferulic acid. It could be said that these drugs had the ability to exert anti-oxidant effect, and restoring structural, hormonal and enzymatic integrity of testicular tissue.

RECOMMENDATION

It is recommended trying to avoid exposure to risk factors e.g. cadmium due to air pollution, tobacco smoking, and consumption of contaminated drinking. If we cannot avoid this we must protect ourselves by using antioxidants in the form of healthy food or antioxidant drugs as a prophylactic measure to minimize the hazardous effects of any environmental risk factor.

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