Ameliorative potentials of a combination of Fenugreek and α-tocopherol on cadmium induced testicular toxicity. An ultrastructural study (Manuscript).pdf

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Ameliorative potentials of a combination of fenugreek and alpha-tocopherol on cadmium induced testicular toxicity: an ultrastructural study

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Background: The current study aimed to elucidate the protective role of combined fenugreek and α-tocopherol against cadmium induced histopathological changes in the testes.

Materials and methods: Thirty adult male albino rats divided into three equal groups 10 rats each. Group I is the control group. Group II received 5 mg/kg/day cadmium chloride. Group III received 5 mg/kg/day cadmium chloride and 150 mg/kg/day fenugreek and 100 mg/kg/day of α-tocopherol. The treatment of all groups was done by oral gavage for 60 consecutive days. The testes were removed and subjected to histopathological and ultrastructure study.

Results: Rats exposed to cadmium showed severe histopathological changes in the testicular spermatogenic series, many vacuoles and multinucleated giant cells. Treatment with fenugreek and α-tocopherol partially improved the morphological changes, reduced tissue damage and rebuilt of the spermatogonia layer.

Conclusions: Fenugreek and α-tocopherol might represent a promising medicinal combination to ameliorate the toxic effects of cadmium exposure. (Folia Morphol 2015; 74, 3: 325–334)

Key words: cadmium chloride, fenugreek, α-tocopherol, seminiferous epithelium, ultrastructure

INTRODUCTION

Recently, cadmium has received global interest as an ecological pollutant primarily because of its immensely long biological half-life, so exposure to cadmium still causes toxic effects even after long periods of a cessation of exposure due to its residual effects [27, 49].

Cadmium (Cd) is a heavy metal used in paint pigments, nickel-cadmium batteries, plastics, dyes, and electrochemistry. It is found in cigarette smoke, fungicides, insecticides, and commercial fertilizers that are used in agriculture [17, 46]. Cd may cause nephrotoxicity, testicular necrosis, prostatic and testicular cancers, liver damage, cardiovascular diseases, osteoporosis and neurodegenerative conditions [59].

Peroxidation induced by Cd is the cause of the release of free oxygen radicals [30]. The oxygen radicals lead to the destruction of sensitive molecules and tissues [39]. After exposure to Cd, testicular damage results and can be observed at the interstitial and tubular levels [59]. Permeability alterations in the capillary endothelium, which cause oedema,
haemorrhages and necrosis, appear to be involved in the histopathological mechanism of these lesions [22]. Moreover, it is well known that prolonged Cd exposure leads to carcinogenic effects on the male reproductive organs [29] and leads to a reduction in reproductive capabilities [48].

Vitamin E is the collective name for a band of fat-soluble substances with unique antioxidant activities that is available in 8 chemical forms (α-, β-, γ- and δ-tocopherol, α-, β-, γ- and δ-tocotrienol) that have diverse degrees of biologic activity [12]. The chemical form α-tocopherol is the most recognised and found in many food items, including nuts, seeds and vegetable oils [21]. The body forms reactive oxygen species (ROS) endogenously which comprise both free radical and non-free radical oxygen such as hydrogen peroxide (H₂O₂), superoxide (O₂⁻) and hydroxyl radical (OH) [57]. Alpha-tocopherol causes the elimination of the free oxygen radicals and the inhibition of the peroxidation [19]. It acts by eliminating lipid peroxyl and alkoxyl radicals, so it suppresses the chain reaction of lipid peroxidation and promotes the production of scavenger antioxidant enzymes [18].

Numerous reports in several labs are going on to evaluate the preventive influence of different natural substances toward poisonous metals [24]. Fenugreek seed (Trigonella foenum-graecum L. cultivar Baladi, family: Leguminosae) is herb of the Legume family that is broadly grown in Egypt and India. Used with meal as a spice and in medication where it demonstrates antioxidant impact in diabetes mellitus due to the existence of various ingredients [20, 53].

The aim of this study is to evaluate the cytoprotective effects of fenugreek and α-tocopherol on Cd chloride induced testicular damage.

**MATERIALS AND METHODS**

Ethical approval. This study was conducted after receiving the approval of the Medical Research Ethics Committee, Faculty of Medicine, King Abdulaziz University.

Animals. Thirty male adult Wistar rats aged 120 days that weighed 175–200 g and were obtained from the animal house were weighed and randomly placed into three groups (n = 10). The rats were housed individually in stainless steel cages with controlled temperature (22 ± 2°C) and humidity (55 ± 10%) and 12/12 h cycle of light and darkness with access to food and drinking water ad libitum. The experimental procedures were performed in accordance with the international guidelines for the care and the use of animals in a laboratory.

**Chemicals.** Cadmium chloride (CdCl₂, 99% pure) and α-tocopherol were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA).

**Fenugreek powder preparation.** Fenugreek (Trigonella foemnum-graecum L.) was purchased from local herbal market. Fifty grams of fenugreek-dried seeds were crushed, boiled in 1 L of distilled water for half an hour. Then the extracts were filtered. The powder was mixed at 5% w/w in standard rat pelleted diet (i.e. 5 g of dry fenugreek in 95 g of rat food). The dose used in the current work was selected according to a previous research [4]. The chemical constituents of fenugreek are carbohydrate (49.81 g%), protein (27.31 g%), fat (7.11 g%), ash (3.67 g%), steroidal saponins (2 g%), flavonoids (399.67 mg%), trigonelline alkaloid (380 mg%) and polyphenols (251.71 mg%) [4, 26].

**Experimental design.** Group I received 1 mL of normal saline and served as the control group. Group II received 5 mg/kg/day CdCl₂ (1/15 LD50) [43]. Group III received 5 mg/kg/day CdCl₂ and 150 mg/kg/day fenugreek and 100 mg/kg/day of α-tocopherol [6]. The prophylactic combination started 1 week before CdCl₂ administration in the current study to acquire the benefits of both curative and preventive regimens. All groups were treated by oral gavage for 60 consecutive days.

Testis histology. At the end of the experiment, the animals were anesthetised, weighed and sacrificed by decapitation. The testes were removed and weighed. Subsequently the testes were fixed in 10% neutral buffered formalin, processed for 5-µm paraffin sections and stained with haematoxylin and eosin (H&E). For each specimen, at least 3 to 5 slides were examined using an Olympus BX53 microscope equipped with a DP73 digital camera (Olympus, Tokyo, Japan) [34, 38, 54].

**Immunohistochemistry.** Paraffin sections were de-waxed in xylene, rehydrated and pretreated with 3% of hydrogen peroxide to block endogenous peroxidase activity. Microwave-assisted antigen retrieval was performed for 20 min. Slides were then incubated overnight at 4°C with the primary antibody (Anti CD68+ for macrophages [1:50], Anti CD3+ for T-lymphocytes [1:200] and Anti CD20+ for B-lymphotyes [1:300]) (Rabbit Polyclonal Antibody from Neo-Markers, Lab Vision Corporation) and sections were incubated with biotinylated IgG and then with streptavidin-peroxidase conjugate (Zymed Corp.).
Sections were then washed with phosphate-buffered saline (PBS) and incubated with 3-3’ diaminobenzidine tetrachloride (DAB) for 5 min to detect immunoreactivity. Sections were counterstained with haematoxylin. Negative control sections were prepared by omitting the primary antibody. Positive control standard laboratory slides were used for all stains to validate the success of the technique. All slides were examined under light microscopy and the presence of labelled cells was documented. The absence of staining was recognised as a negative result (−) while the presence of brown staining was recognised as a positive result (+) [54].

**Morphometry.** Five slides of the testes of each group (6 fields per slide), seminiferous tubules round or almost round were selected indiscriminately and analysed. The mean seminiferous tubule diameter was obtained by measuring across the minor and major axes. The seminiferous epithelium height was measured for the same tubules. The epithelium height 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 value [33]. The measurements were taken at magnifications of ×100 and ×200 using the Image-Pro Plus v6.0 (Media Cybernetics, Maryland, USA) and an Olympus BX53 microscope. In addition, the percentage of the immunopositive cells in the testicular tissue was evaluated.

**Ultrastructure study.** Testes samples of approximately 1 mm³ were obtained and immersed in 2.5% of glutaraldehyde in a 0.1 M buffer phosphate at 4°C for 3 h and post-fixed in 1% of osmium tetroxide. After dehydration in a graded series of ascending concentrations of ethanol, the tissues were embedded in epon 812. Semithin sections were prepared from the blocks, stained using toluidine blue and observed with the Olympus microscope. Demonstrative areas of semithin sections were chosen. Ultrathin sections of a 50–60 nm of thickness were cut using ultramicrotome (NOVA, LKB 2188, Bromma, Sweden) and uranyl acetate and lead citrate were used to stain the tissues and then inspected using a Philips 201 transmission electron microscope at 60–80 kV in a transmission electron microscope unit (Philips Industries, Eindhoven, Netherlands) [37].

**Statistical analysis.** Quantitative data were expressed as the mean ± standard deviation of different parameters for the treated groups. The data were analysed using the one way analysis of variance (ANOVA) after testing Levene’s test verified the equality of variances in the samples (homogeneity of variance) (\( p > 0.05 \)). In the parametric data which equality was assumed we used Tukey’s post-hoc test for comparison between variable, meanwhile in parametric parameters in which equality of data were not assumed (CD3, CD20, CD68) we use Dunnett’s T3 test for comparison between groups. The statistical analysis was performed using SPSS version 22. The values were considered significant when \( p < 0.05 \).

## Results

### Body and testes weight.

Rats received \( \text{CdCl}_2 \) revealed a significant reduction in the body weight and testis weight in comparison with control group. Administration of combined fenugreek and \( \alpha \)-tocopherol with \( \text{CdCl}_2 \) improved body weight and testes weight (Table 1).

### Table 1. Mean testes weight and body weight

<table>
<thead>
<tr>
<th></th>
<th>Body weight [g]</th>
<th>Testis weight [g/100 g]</th>
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<tbody>
<tr>
<td>Control (( n = 10 ))</td>
<td>210.40 ± 3.062</td>
<td>0.73 ± 0.036</td>
</tr>
<tr>
<td>( \text{CdCl}_2 (n = 10) )</td>
<td>199.5 ± 0.028*</td>
<td>0.61 ± 0.084*</td>
</tr>
<tr>
<td>( P &lt; 0.001 )</td>
<td></td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td>Combined fenugreek and ( \alpha )-tocopherol with ( \text{CdCl}_2 (n = 10) )</td>
<td>208.10 ± 0.994</td>
<td>0.68 ± 0.030</td>
</tr>
<tr>
<td>( P &lt; 0.128 )</td>
<td></td>
<td>( P &lt; 0.139 )</td>
</tr>
</tbody>
</table>

*Values are expressed as a mean ± standard deviation. The analysis was conducted using one-way ANOVA followed by Tukey’s post-hoc test.
sloughed, detached and lost the support of Sertoli cells so they could no longer survive and there was appearance of giant cells with multiple nuclei (Fig. 1B, C). Combined fenugreek and α-tocopherol with CdCl$_2$ minimised the degenerative changes evidenced by the reappearance of the spermatogonia near the basal lamina as well as the restoration of the structure of the seminiferous tubules and no cavities or vacuoles were detected nearly in all exposed animals (Fig. 1D).

Toluidine blue stained sections. The testes of the control group reveal the typical histological picture of the seminiferous tubules (Fig. 2A). The group treated with CdCl$_2$ showed disorganisation of the seminiferous tubules with an obvious diminution of the epithelial layers, extensive degenerative vacuolisation and intercellular empty spaces in between the cells. Also, the spermatozoa disappeared, the spermatids exfoliated, the primary spermatocytes’ density was diminished and the spermatogonia noticeably deteriorated and depleted (Fig. 2B, C). Combined fenugreek and α-tocopherol with CdCl$_2$ showed an apparent improvement of the architecture of the seminiferous tubules with a partial restoration of a low germinal epithelium more or less in all exposed animals and minimal or no vacuolations among the germ cells (Fig. 2D, E).

Morphometric analysis. The mean seminiferous tubular diameter showed variations in the different groups in comparison with the control group. The data showed that there was a significant decrease in the mean diameter of the seminiferous tubules in the CdCl$_2$ treated group in comparison with the control group, however there was an improvement in the diameter of the seminiferous tubules of the group treated with combined fenugreek and α-tocopherol and CdCl$_2$. The mean germin:al epithelial height of the CdCl$_2$ treated group showed a significant decrease in comparison with the control group. The group treated with CdCl$_2$ and combined fenugreek and α-tocopherol showed significant improvement in the epithelial heights (Table 2).

Immunohistochemistry study. The data showed that there were significant increases in the area percentage of CD3, CD20 and CD68 in the CdCl$_2$ treated group in comparison with the control group (Table 3).

Ultrastructure picture. The transmission electron micrographs of the control group reveal a normal structural organisation of the seminiferous tubules and the interstitium. The CdCl$_2$-treated testes revealed several vacuoles of variable sizes inside and in between the germ cells and an apparently thickened basal lamina with a myoid cell. Sertoli cells with an irregular euchromatic nucleus and the characteristic indentation of the nucleus resting on the basal lamina with numerous vacuoles and an increase in the density of the cytoplasmic inclusions, also unhealthy damaged cells were observed. The primary spermato-
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Mid-piece of the sperm with distorted and swollen mitochondrial sheaths surrounds the central axoneme. Spermatids with rounded or oval nuclei that have disrupted chromatin and peripherally arranged cytes with disrupted chromatin are separated by the variable sizes of the intercellular spaces and the vacuolated distorted mitochondria. In addition, several vacuoles of variable sizes all over the cytoplasm were observed. Mid-piece of the sperm with distorted and swollen mitochondrial sheaths surrounds the central axoneme. Spermatids with rounded or oval nuclei that have disturbed chromatin and peripherally arranged

Table 2. Mean diameter of seminiferous tubules (MSTD) and germinal epithelial height

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 200)</th>
<th>CdCl₂ (n = 200)</th>
<th>Combined fenugreek and α-tocopherol (n = 200)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular diameter (MSTD) [µm]</td>
<td>253.427 ± 17.536</td>
<td>240.256 ± 29.926</td>
<td>246.223 ± 34.145</td>
</tr>
<tr>
<td>Seminiferous epithelium height [µm]</td>
<td>76.502 ± 5.549</td>
<td>70.066 ± 8.319</td>
<td>73.333 ± 7.962</td>
</tr>
</tbody>
</table>

P < 0.0001* P < 0.029* P < 0.0001* P < 0.0001*

*Values are expressed as a mean ± standard deviation. The analysis was conducted using one-way ANOVA followed by Tukey’s post-hoc test.

Table 3. Mean area percentage of CD3, CD20, and CD68 immunostaining

<table>
<thead>
<tr>
<th>Area %</th>
<th>Control (n = 10)</th>
<th>CdCl₂ (n = 10)</th>
<th>Combined fenugreek and α-tocopherol (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>0.031 ± 0.027</td>
<td>0.090 ± 0.049</td>
<td>0.056 ± 0.060</td>
</tr>
<tr>
<td>P &lt; 0.014*</td>
<td></td>
<td></td>
<td>P &lt; 0.553</td>
</tr>
<tr>
<td>CD20</td>
<td>0.050 ± 0.022</td>
<td>0.091 ± 0.040</td>
<td>0.061 ± 0.026</td>
</tr>
<tr>
<td>P &lt; 0.033*</td>
<td></td>
<td></td>
<td>P &lt; 0.692</td>
</tr>
<tr>
<td>CD68</td>
<td>0.058 ± 0.028</td>
<td>0.139 ± 0.080</td>
<td>0.106 ± 0.073</td>
</tr>
<tr>
<td>P &lt; 0.034*</td>
<td></td>
<td></td>
<td>P &lt; 0.213</td>
</tr>
</tbody>
</table>

*Values are expressed as a mean ± standard deviation. The analysis was conducted using one-way ANOVA followed by Dunnett’s T3 test post-hoc test.
the mitochondria with lost cristae and acrosomal cap. Leydig cells with oval, indented nuclei with disrupted chromatin are noticed and apparent electron dense bodies, vacuoles, empty spaces, lymph vessels and ruptured vacuolated mitochondria. Unhealthy cells characterised by heterochromatin condensation and shrinkage of both cytoplasm and nucleus, which is an indication of phagocytosis (Fig. 3A–D).

While the recovery group showed minimal vacuolation, some mitochondria were still affected and others are healthy mid pieces of the sperm were nearly improved and restored nearly in all exposed animals. Reconstruction of the structure of the cross-sections of the mid pieces with axoneme surrounded by a mitochondrial sheath is noticed. Leydig cells with oval euchromatic nuclei and peripherally located heterochromatin. The cytoplasm contains multiple lysosomes. Improvement of the appearance of germ cells is observed. The Spermatids with rounded or oval euchromatic nuclei and peripherally arranged mitochondria with preserved cristae and acrosomal cap were observed. Minimal empty spaces in the affected intercellular bridge are still noticed (Fig. 4A–D).

**DISCUSSION**

Cadmium is a poisonous heavy metal that is attributed to it reduced elimination rate [51]. Male genital organs are obviously affected during environmental exposure to Cd as proved by bad quality of seminal fluid and sterility [7]. In the current study, it is observed that administration of CdCl₂ lowered testicular weight and body weight and this is related to reduced androgen level [52]. Moreover, CdCl₂ decreases total sperm count and raises anomalous sperm forms [35] and induces necrotic destruction of testicular tissues [25]. In addition, the undifferentiated spermatogenic cell count is declined [40].

With light microscopic examination, CdCl₂ treated group showed degenerative alterations in the testicular histology with damage of the supporting cells of Sertoli that ultimately causes irreversible loss and detachments of spermatogonia and this agreed with...
Morel et al. [36] found that Leydig cells’ morphology is altered with disrupted chromatin followed by reduction in the cell number and is irreversibly substituted by inflammatory cells and this agreed with the current findings that are supported by the immunomarkers increased expression. It is noted that fenugreek consumption restored serum testosterone level and testicular weight [45]. The fenugreek being an antioxidant, it performs a crucial role in restoring testosterone level by eliminating ROS which is known to stop steroidogenesis [45, 60].

The cell type that is damaged is mainly in the early stage of spermatogenesis and the degree of germ cell damage is related to the dose of Cd exposure [9]. This is consistent with the current findings that the spermatogonia are the primary cells that are affected. In contrast, another researcher observed that the exfoliated sloughed germ cells were spermatocytes and spermatids [44].

Administration of Cd in the present study produced atrophy, vacuolations, cellular debris and the seminiferous epithelium was sloughed off at many points. These changes are consistent with the findings of the author who demonstrated that a single dose of Cd induced severe necrosis and degeneration of the seminiferous tubules with a complete loss of the centrally located spermatozoa [2].

In the current research, a wide interstitium and congested capillaries were noticed. Burukoğlu and Bayçu [10] observed several congested thickened blood vessels and homogeneous oedematous material in the interstitium which is infiltrated by different cells such as leukocytes and macrophages. The thickened blood vessel wall and oedema are due to cytokines secreted by macrophages situated in the peritubular zones [1, 44].

Ultrastructurally, the changes of the thickness of basal lamina observed in the present research could be explained by the study of Shokri et al. [50] who found that certain stimulants might encourage the myoid cells to yield more collagen and extracellular matrix, which cause the thickening of the basal lamina.
In the current work, numerous intercellular areas were noticed in-between the germinal cells lining the seminiferous tubules. This is attributed to loosening of cell–cell connections and these gaps caused shrinkage of both germ and Sertoli cells [14]. A previous study observed disarrangement of germ cell structure and function that is due to elevated level of ROS in the membrane lipids [16, 56].

In CdCl₂ treated group, Sertoli cells showed irregular euchromatic nuclei with variable damage of germ cells [23] and this agreed with the current findings. The present study revealed ultrastructural alteration of sperms as shown in the transverse sections of the mid-pieces of sperm that revealed disrupted axoneme and a distorted swollen mitochondrial sheath.

The mechanism of Cd induced injury involves the production of free radicals, which modify mitochondrial activity [13]. Therefore, other authors have postulated that antioxidants could be a critical aspect of the efficient management of Cd intoxication [11, 16].

Current work revealed that fenugreek administration reduces the unhealthy consequences of Cd on testicular tissues that are attributed to enhancement of antioxidant potential due to minimising lipid peroxidation [28]. Moreover, an experimental research demonstrated that consumption of herbal substance that contains polyphenols and flavonoids exerts antioxidant role and suppresses low density lipoprotein oxidation [55].

Some researchers have discussed the immunohistochemistry of the testicular architecture and they observed that macrophages and lymphocytes were limited around the basal lamina and in the interstitial spaces [32]. Other investigator observed that immune response infiltration of monoclonal antibodies for B- and T-lymphocytes and macrophages occasionally occurred in the lumen of the testes [26]. This is in accordance with our findings that CD68 immunoreactivity (macrophages) showed significant increase that is improved with the administration of combined fenugreek and α-tocopherol. In the current study, CD20 showed significant increase in immunohistochemical positivity with Cd and this distinguishes cell injury, degeneration, inflammatory conditions and death of components of the testes [31]. A researcher noticed that crucial aetiological element responsible for male infertility is infection and inflammation of the epithelial tissue and suggested that T-lymphocytes participate in the regulation of male infertility pathogenesis [15].

A previous research suggests that fenugreek can be regarded a potential therapeutic nutrient to guard against male reproductive toxicity caused by Cd through its antioxidant, anti-inflammatory and antifibrotic actions [5].

A study using α-tocopherol and Cd showed the seminiferous tubules and the Leydig cells almost retained their normal structure with normally arranged the seminiferous epithelium and a healthy interstitium [41]. Most of the mid-pieces of the sperm had a normal structure except a few of them showed distorted and swollen mitochondrial sheaths [41] and this agreed with the current findings.

Alpha-tocopherol has been found to minimise infections [8] and activate the adaptive immune system which was detected by the expression of B-lymphocytes and T-lymphocytic surface antigens [36]. The antioxidant α-tocopherol has also been found to be capable of diminishing apoptosis in degenerating cells of the testicular tissues [42]. Therefore, the role of α-tocopherol can be defined as a defensive role for the testicular epithelium and lymphocytic system.

Our findings revealed that combined fenugreek and α-tocopherol with CdCl₂ decreases earlier structural alterations and reduces the injury and this coincides with the findings of a prior research [58]. The ameliorating effects of combined fenugreek and α-tocopherol in the current work were slight and these results are consistent with the progression of alterations in the rat testes as a result of Cd intoxication explained by Sen Gupta et al. [47]. Alpha-tocopherol supplement has demonstrated fruitful effect in enhancing sperm quality [3]. This agreed with our suggestion that supplements of combined fenugreek and α-tocopherol alleviates the oxidative stress. This regimen could re-establish the activity of antioxidant enzymes to allow regular germ cell differentiation and guard sperm count and protect testicular morphology throughout Cd exposure.

**Limitations of the study**

The limitations of the study are due to the small number of animals so, we advise to apply this study on a larger number of animals and different spices to facilitate the statistical analysis and to broaden the feedback. Also, we advise to apply different doses of the combination of fenugreek and α-tocopherol. Quantitative measures as stereology are recommended to support the hypothesis of the study.
CONCLUSIONS

The results obtained from the current study reveal that fenugreek and α-tocopherol ameliorate histopathological variations of the testicular tissues created by Cd to some extent. These combined substances might be used as a dietary consumption of humans who are exposed to environmental toxins especially Cd.

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