

Effect of fenugreek seed extract on carbofuran-inhibited spermatogenesis and induced apoptosis in albino rats

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Abstract

Carbofuran is a broad spectrum carbamate pesticide used against different pests. Fenugreek (*Trigonella foenum graecum*) is used as a medicinal plant and showed many therapeutic effects. The present work studied the effect of aqueous extract of fenugreek seeds on carbofuran induced testicular toxicity in albino rats. Treating rats with carbofuran for 6 weeks induced significant decrease in testis weights, diameters and germinal epithelial heights of the seminiferous tubules. Histological results revealed intertubular hemorrhage, degeneration of the interstitial tissue and reduction of spermatogenic cells. Expression of caspase-3 and bax increased in the vgerm cells. Biochemical results showed decrease of testosterone and LH in sera of the treated animals. Coadministration of carbofuran with fenugreek seeds extracts ameliorates the histopathological alterations caused by carbofuran in testes of albino rats. Moreover, it caused decrease of apoptosis as indicated by decrease of expression of caspase-3 and bax, and increased testosterone and LH. It is concluded that the effect of fenugreek against testicular toxicity of carbofuran may be due to the antioxidant activity of its constituents (e.g. flavonoids and polyphenols).

Keywords: Carbofuran, Fenugreek, Testis, Rat, Caspase-3, Antioxidants

1. Introduction

The widespread use of chemical pesticides in public health and agriculture has caused severe environmental pollution and health hazards due to its persistence in the food chain (1). Carbofuran (2, 3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a broad spectrum carbamate pesticide commonly used as an insecticide, nematocide and acaricide in agricultural practice throughout the world. It is used against soil and foliar pests of field, fruit, vegetable, and forest crops (2). Carbofuran is a potent cholinesterase inhibitor and is highly toxic by inhalation and ingestion (3).

Pant et al. (4) reported that carbofuran disrupts testicular morphology and alters activities of enzymes associated with specific cell types of testes in rats. It also decreased libido and sperm number in rabbits (5).

In utero or lactational exposure of carbofuran to male rats have caused testicular and spermatotoxicity (6). El-Amoudi (7) reported that carbofuran inhibited spermatogenesis and decreased sperm count in mice.

The use of plants and their extracts in medicinal proposes has been rapidly increasing worldwide. Fenugreek (*Trigonella foenum graecum*) is a herb belongs to family leguminosa. Its seeds are used in many oriented countries as a spice in food preparations due to their strong flavor and aroma (8). It is also used as herbal medicine for their carminative, tonic and aphrodisiac effects (9). Fenugreek seeds exhibit hypoglycemic, hypolipidaemic, antifertilitic, antiandrogenic, antinociceptive and wound healing properties and are good source of dietary fibers (10). Recently, Sakr et al. (11) reported that fenugreek seeds prevent adriamycin-induced cytogenetic and testicular damage in albino rats. The present study was undertaken to demonstrate the effect of fenugreek seeds extract on testicular toxicity induced by carbofuran in rats.

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2. Materials and Methods

2.1. Fenugreek aqueous extract

Dried and fresh batches of fenugreek seeds were purchased from local market. Seeds were washed with distilled water to get rid of extraneous matter, air-dried and ground into a fine powder in a mixer. The powder was mixed with distilled water (1 g of seed powder per 100 ml) in a vortex cyclomixer for 10 minutes and then centrifuged at 10000 rpm and the supernatant was collected. The supernatant was used as the aqueous extract for feeding the animals and was freshly prepared. In this study, each animal was orally given 1 ml of the final aqueous extract containing 0.4g/kg body weight fenugreek seeds (11).

2.2. Animals

Male Wistar rats weighting 180 ± 10 g were obtained. They were kept in the laboratory under constant temperature ($24 \pm 2^\circ\text{C}$) throughout the experimental work. They were maintained on a standard rodent pellets and water was available *ad libitum*. All the experiments were done in compliance with the guide for the care and use of laboratory animals. Animals were divided into 4 groups:

Group I. These animals (10 rats) served as controls.

Group II. Animals in this group were orally given 1 ml of final aqueous extract of 0.4 gm/kg fenugreek 3 times weekly by gastric intubation for 6 weeks.

Group III. Animals of this group (25 rats) have been orally given 0.1 ml of saline contains 2 mg/kg body weight carbofuran, 3 days weekly for 6 weeks.

Group IV. Animals of this group (30 rats) have been orally given carbofuran (2mg/kg body weight), followed by fenugreek extract 3 days weekly for 6 weeks.

2.3. Histological study

Immediately after decapitation animals were dissected, testis were removed from treated and control animals and fixed in Bouin's solution. After fixation, specimens were dehydrated in an ascending series of alcohol, cleared in two changes of xylene and embedded in molten paraffin. Sections of 5 microns thickness were cut using rotary microtome and mounted on clean slides (12). For histological examination, sections were stained with Ehrlich's haematoxylin and counterstained with eosin. Seminiferous tubules diameter and germinal epithelial height were measured from the spermatogenic cells on the inner surface of the basement membrane through the most advanced cell types lining the lumen of the tubules.

2.4. Immunohistochemical Study

From each testis block, 4 microns thick sections were cut on Neoprene-coated slides. The immunostaining was performed using the avidin biotin complex (ABC) method and an automatic autostainer (CODE-ON Immuno/DNA slide stainer: Biotek solution, Santa Barbara, CA). Slides were deparaffinized and blocked for endogenous peroxidase with 1 .75% hydrogen peroxide in methanol for 20 mm, antigen retrieval for 15 mm using Biogenex Antigen Retrieval Citra solution in 90°C water bath for 30 mm. The slides were allowed to cool for 20 min before continuing. Slides were then blocked by normal bovine serum for 5 mm at 37°C . The monoclonal antibody was applied overnight in humid medium at room temperature followed by the biotinylated secondary antibody for 15 min at 37°C and the ABC complex for 15 min at 37°C (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) was applied for 20 min at room temperature as chromogenic slides were counterstained with hematoxylin, dehydrated, and covered by coverslips. In negative control slides, the same system was applied with replacement of the monoclonal antibody by diluted normal bovine serum. Anti-caspase-3 and anti-bax (Dako, Cambridge, UK) monoclonal antibody was used for detection of caspase and bax, respectively.

2.5. Biochemical study

For biochemical assays determination, blood samples were collected from the inferior vena cava and then centrifuged. Sera were obtained by centrifugation of the blood sample and stored at -20°C . Testosterone and LH were determined using radioimmunoassay kits supplied by Diagnostic Products Corp. (Los Angeles, CA, USA) according to Maruyama et al. (13).

2.6. Statistical Analysis

Data were expressed as mean values $\pm$ SD and statistical analysis was performed using one way ANOVA to assess significant differences among treatment groups. The criterion for statistical significance was set at $P < 0.05$. All statistical analyses were performed using SPSS statistical version 16 software package (SPSS® 4 Inc., USA).

3. Results

3.1. Morphometric results

Results in table 1 revealed that treatment with carbofuran caused significant decrease in testis

weight. The diameter of seminiferous tubules as well as germ cell height was significantly decreased in carbofuran treated animals. Animals treated with carbofuran and fenugreek seed extract showed marked improvement in the mean testes weights, tubular diameter and in germ cell height in comparison with carbofuran treated animals.

3.2. Histological results

Examination of testis of control rats showed typical features of normal seminiferous tubules, spermatogenic cells, intertubular connective tissue and spermatozoa (Fig. 1a). Animals given fenugreek seeds extract showed normal histological structure. Treating rats with carbofuran exhibited a distinct

histological differences. After 4 weeks, intertubular hemorrhage was seen and the spermatogonia appeared vacuolated with pyknotic nuclei (Fig. 1b). After 6 weeks, the seminiferous tubules were markedly separated from each other and the interstitial tissue were degenerated. The germ layers reduced and the spermatogenic cells degenerate. Sperm bundles were completely absent in the tubules and many degenerated cells were exfoliated in their lumens (Fig. 1c).

Examination of testes of animals given carbofuran and fenugreek extract showed less histopathological changes and most of the tubules appeared to contain more or less normal germ layers and sperm bundles (Fig. 1d).

Tble 1. Mean values of the tests weight, diameter and epithelial height of seminiferous tubules in rats treated with carbofuran and fenugreek seeds

Animal group	Testis weight (g)	Diameter of seminiferous tubules (μm)	Epithelial height of seminiferous tubules (μm)
Control	1.6 $\pm$ 0.2	240 $\pm$ 5.5	99 $\pm$ 4.5
Fenugreek	1.63 $\pm$ 0.11	248 $\pm$ 4.5	102 $\pm$ 3.6
Carbofuran	1.32 $\pm$ 0.1*	188 $\pm$ 12.2*	78 $\pm$ 3.2*
Carbofuran $\pm$ Fenugreek	1.45 $\pm$ 0.2	204 $\pm$ 8.6*	91 $\pm$ 2.6*

* Significant at P < 0.05 in comparison with controls

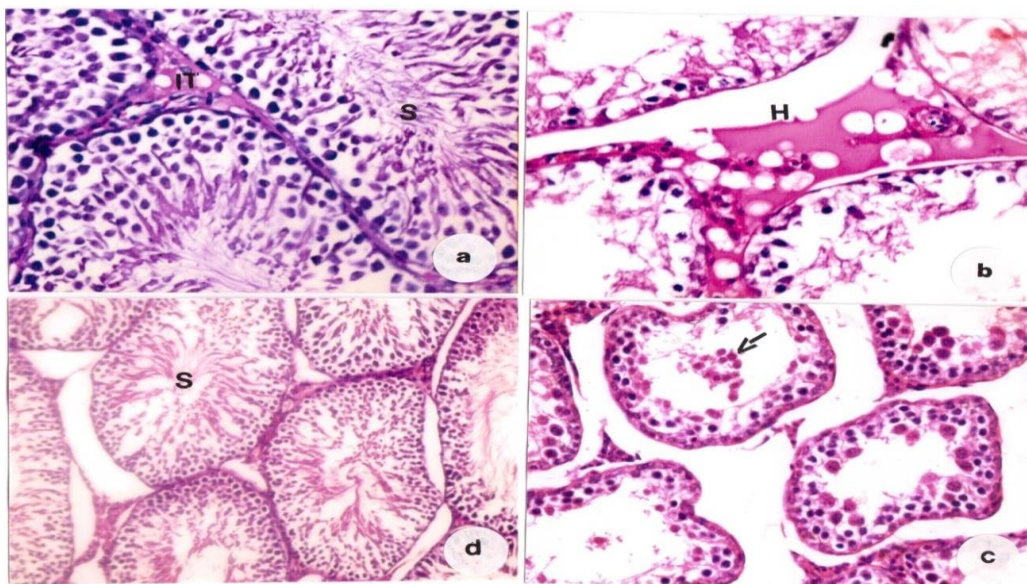


Figure 1. (a) Section in testis of a control rat showing normal seminiferous tubules; S: sperms, IT: interstitial tissue. (b) Section in testis of a rat treated with carbofuran showing intertubular hemorrhage (H). (c) Section in testis of a rat treated with carbofuran showing loss of germ layers and exfoliation of cells in the lumen of the tubules (arrow). (d) Section in testis of a rat treated with carbofuran and fenugreek showing increase of spermatogenic cells, S: Sperms X300.

3.3. Immunohistochemical results

Figure 2 (a & b) showed the expression of Bax in Leydige cells. The number of the Bax positive staining cells increased in Leydige cells of rats treated with cabofuran compared with control and decreased after treatment with carbofuran and fenugreek extract (Fig. 3). Immunohistochemical examination of testes of carbofuran-treated rats revealed that caspase-3 expression increased in germ cells (spermatogonia, primary and secondary spermatocytes) (Fig. 4a). Treating animals with carbofuran and fenugreek extract showed a decrease of caspase expression (Fig.4b). The percentage of caspase-3 expression was 8% in control rats

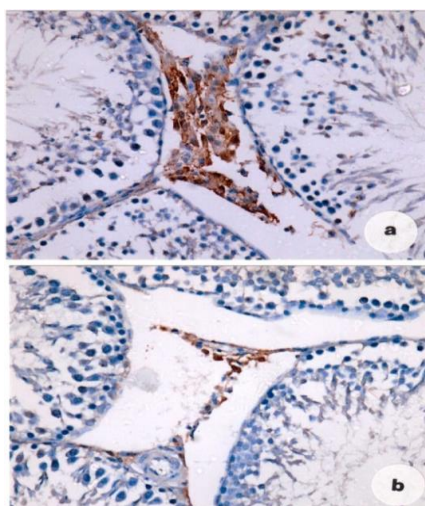


Figure 2. Section in testis of a rat treated with carbofuran showing increase of Bax expression in Leydig cells (b) Section in testis of a rat treated with carbofuran and fenugreek showing decrease of Bax expression, X300.

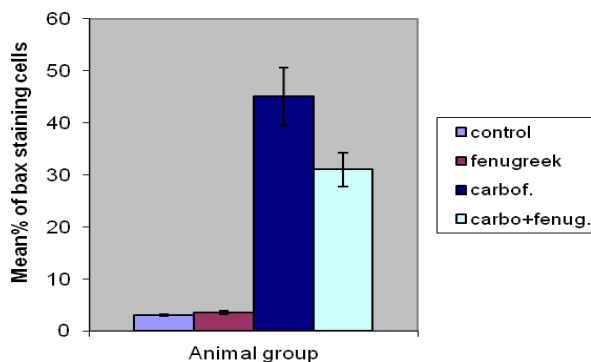


Figure 3. Mean % of bax staining cells in different animal groups.

compared with 37% and 19% in rats intoxicated with carbofuran and carbofuran and fenugreek extract, respectively (Fig. 5).

3.4. Biochemical results

The control and fenugreek-treated rats had equivalent testosterone and LH levels at the end of the 6th week. Treating animals with carbofuran caused a significant decrease ($P<0.05$) in levels of testosterone and LH. On the other hand, coadministration of carbofuran with fenugreek seeds extract caused an increase in serum levels of these two hormones (Fig.6 a&b).

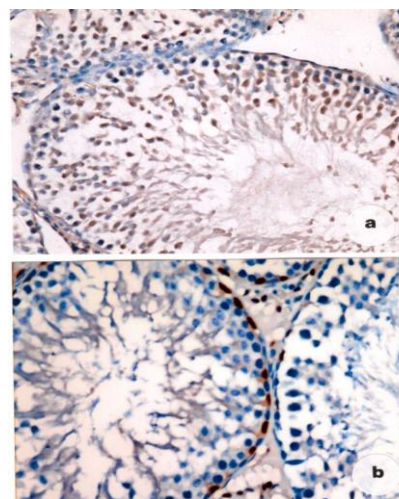


Figure 4. Section in testis of a rat treated with carbofuran showing increase of caspase-3 expression in germ cells (b) Section in testis of a rat treated with carbofuran and fenugreek showing decrease of caspase-3 expression, X300.

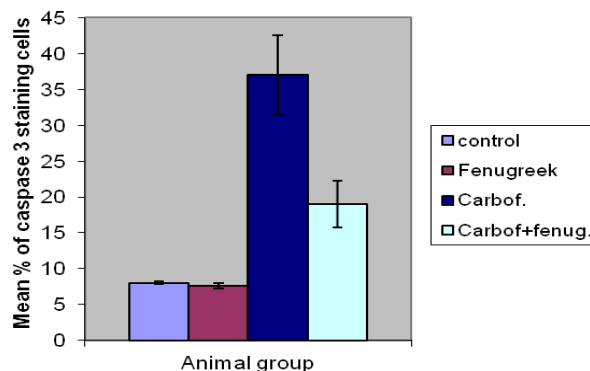


Figure 5. Mean % of caspase-3 staining cells in different animal groups.

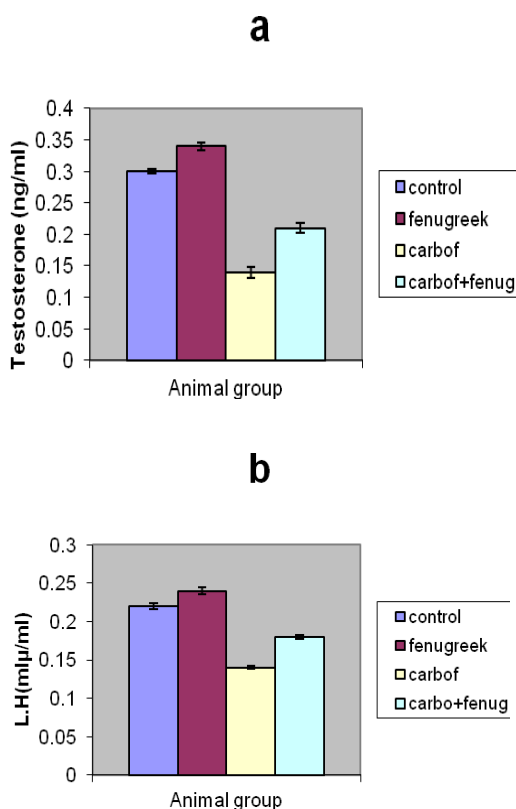


Figure 6. Change in testosterone (a) and LH (b) in different animal groups.

5. Discussion

The use of chemical insecticides resulted in lethal effects on non-target organisms and has direct toxicity to users. The present results showed that intoxicated rats with carbofuran resulted in significant decrease in testes weights, seminiferous tubule diameter and germinal epithelial height. Histological observations revealed intertubular hemorrhage, degeneration of spermatogenic cells as well as the interstitial tissue. Moreover, a reduction of germ layers was recorded. Similar results were obtained in mammalian animals exposed to carbofuran (5,6,14). Recently, El-amoudi (7) reported that carbofuran inhibited spermatogenesis and decreased sperm count in mice. The spermatogenesis in mammals depends on testosterone production by the interstitial Leydig cells (15). The obtained results showed decrease of testosterone and LH in sera of carbofuran treated rats. Similarly, Goad et al., (16) found that exposure of rats to sublethal amounts of carbofuran decreased testosterone, while the levels of progesterone, cortisol, and estradiol were significantly increased. Thus, the effect on germ cells

might be due to the decrease in testosterone level resulting from exposure to carbofuran.

The present study revealed that carbofuran induced testicular apoptosis as indicated by increase of caspase-3 and bax in germ cells. Similar results were obtained in testes of animals exposed to insecticides. Calviello et al., (17) reported that carbamate derivatives such as mancozeb have also been shown to induce oxidative stress, DNA damage and activation of the mitochondrial pathway of apoptosis. Song et al. (18) showed that one,1-dichloro-2,2 bis(p-chlorophenyl) ethylene (p,p'-DDE) could activate apoptosis of cultured rat Sertoli cells in a pro-oxidant and mitochondria dependent manner by activating the intrinsic programmed cell death pathway. Shi et al., (19) reported that in p,p'-DDE exposed rats, apoptosis of Sertoli cells and germinal cells is mediated by a terminal executioner, caspase3, thereby disturbing the spermatogenic process. Time-dependent elevations in the levels of Fas, FasL and caspase-3 were observed in Sertoli and peritubular germ cells of rats exposed to linden (20). Administration of methoxychlor resulted in a significant increase in the levels of cytochrome C, caspase 3 and procaspase 9, Fas-FasL and NF-kappaB in testis of rats (21). Sakr and El-amoudi (22) reported that Bax expression was increased in Leydig cells and p53 expression increased in spermatocytes of testes of deltamethrin treated rats.

Oxidative stress results from excessive biosynthesis or intake of pro-oxidants, impaired biosynthesis of antioxidants, or a combination of both. Balancing pro-oxidants and antioxidants is vital for normal testis function and sperm fertilization ability. Environmental contaminants are capable of elevating ROS levels and depleting ROS-scavenging antioxidants. By inducing oxidative imbalance, these compounds alter key processes, such as apoptosis, spermatogenesis and steroidogenesis. Carbofuran is known to generate reactive oxygen species (ROS) and result in oxidative stress in intoxicated animals (23). Thus, carbofuran may induce oxidative stress and resulted in the recorded histomorphological alterations and apoptosis in testes of albino rats involving expression of caspase-3 and Bax.

In the present study, it was found that fenugreek seeds extracts ameliorates the histopathological alterations caused by carbofuran in testes of albino rats. Moreover, it caused decrease of apoptosis as indicated by decrease of expression of caspase-3 and bax. Consistent with these findings, Lamfon (24) showed that oral administration of fenugreek seeds extract improved the histological changes induced by carbendazim and suppress the oxidative stress as

indicated by decrease of lipid peroxidation and increase activity of SOD and CAT. Sakr et al., (11) reported that fenugreek seeds extract ameliorate adriamycin induced testicular toxicity and oxidative stress in mice. When diabetic rats were treated with aqueous extract of fenugreek seeds, marked recovery of testis and well-developed spermatogenic activity and Leydig cells were seen (25, 26).

Fenugreek seeds are rich source of many active phytochemicals such as saponins, coumarin, fenugreekine, nicotinic acid, saponinins, phytic acid, scopoletin, and trigonelline, which are thought to account for many of its presumed therapeutic effects (27). The antioxidant effect of fenugreek seeds extracts was studied by several investigators. Kaviarasan et al., (28) reported that fenugreek seed polyphenols prevented oxidative hemolysis and lipid peroxidation induced by H_2O_2 *in vitro* in human erythrocyte. Ravikumar and Anuradha (29) found that feeding alloxan-treated diabetic rats with diet containing fenugreek seeds led to a reduction in biomarkers of oxidative damage. Choudhary et al., (30) showed that fenugreek seeds could modulate the activities of SOD, CAT, and glutathione-S-transferase. Fenugreek seed polyphenols proved to be an effective antioxidant and an anti-inflammatory agent in protecting sheep RBCs from membrane damage due to high glucose or increasing H_2O_2 concentrations induced oxidative stress (31). Co-administration of fenugreek seed powder at 5% in pellet diet during two months succeeded to antagonize the harmful effects of $AlCl_3$ and decreased lipid peroxidation in rats (32).

In conclusion, carbofuran caused testicular damage and apoptosis in albino rats and fenugreek seed extract showed ameliorative effect against carbofuran toxicity. This effect may be due to its higher contents of antioxidant compounds (flavonoids and polyphenols).

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