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PROTECTIVE EFFECT OF *OCMUIM BASILICUM* LEAVES EXTRACT ON DIAZINON-INDUCED REPRODUCTIVE TOXICITY AND OXIDATIVE STRESS IN ALBINO RATS

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ABSTRACT

Objective: To evaluate the ameliorative role of the Basil (*Ocmuim basilicum*) leaves on the testicular damage induced by the organophosphorus insecticide, diazinon in albino rats. **Methods**: Animals were divided into four groups. Group I was considered as control. Group II was orally given aqueous O. *basilicum* extract at a dose level of 20 mg/ kg 5 days / week for 6 weeks. Group III was orally given diazinon at a dose of 20mg/ kg.b.wt, 5 days / week for 6 weeks. Group IV: rats were given diazinon followed by oral administration with aqueous *O. basilicum* extract 5 days/ week for 6 weeks. Animals were sacrificed after 3 and 6 weeks of treatment. Testes were removed and stained with H & E for histological examination and nuclear Ki-67 and bax were demonstrated. MDA, SOD and CAT activity in testes of different groups was evaluated. **Results:** Diazinon treatment caused many histopathological alterations, inhibition of spermatogenesis and morphometric changes in the testes. Immunohistochemical changes were detected as strong expression of bax in Leydig cells and decreased expression of Ki-67 in nuclei of spermatogonia. Lipid peroxidation marker, MDA was increased while the antioxidant enzymes namely SOD and CAT were decreased. Treating animals with diazinon and O. *basilicum* extract caused a reduction in testicular alterations caused by diazinon. Moreover, MDA decreased and SOD and CAT increased. **Conclusion**: The results of this study indicated that *O. basilicum* leaves extract lessens the reproductive alterations induced by diazinon in albino rats and this is may be due to the potent antioxidant effects of its components.

KEYWORDS: Diazinon, *Ocmuim basilicum*, Testis, Histology, Oxidative stress.

INTRODUCTION

Pesticides are manufactured organic compounds that are used to control harmful organisms. Contact with these pesticides is an important health issue for agricultural workers. A variety of pesticides can accumulate in animals bodies which concentrate them and pass from pray to predator.[1] Organophosphates are widely applied insecticides in plant protection programs. Diazinon, (0, 0-diethyl 0-(2-isopropyl 1-6-methyl 1-4pyrimidinyl) phosphoorthiate) is an organophosphorus insecticide utilized broadly by commercial and home applicators in a variety of formulations to control different insect pests of ornamental plants and food crops (especially corn, rice, onions and sweet potatoes), nematodes and soil insects in turf, lawns and croplands.[2] Diazinon exerts its toxic effects by inhibiting cholinesterase in many different animals. The toxicity of diazinon was reported in domestic chickens[3], domestic ducklings and goslings[4], laboratory monkey[5] and mice.[6]

The utilization of plants has expanded significantly, in light of the fact that they are turning into a mainstream elective treatment in diverse nations. Plants constitute an imperative wellspring of dynamic regular items with diverse organic properties. *Ocimum basilicum* (Basil) is a yearly herb of the *Lamiaceae* family broadly developed in different locales of the world. The therapeutic and medicinal values of basil are the subjects of many researches. O. *basilicum* leaves extracts were reported to have numerous pharmacological activities including anti-aging, anticancer, antiviral, and antimicrobial properties.[7,8,9]

Supplementation of *O. sanctum* leaf reduced the severity of hydropericardium, hepatitis, myocarditis, oedema in lungs, lymphocytic depletion in lymphoid organs and focal interstitial nephritis.[10] Cholesterol, low-density lipoprotein and triglyceride were reduced after utilization of basil.[11] Rupert[12] reported that basil or basil oil can be used in prevention and treatment of cardiovascular disease. Recently, Sakr and Nooh[13] reported that *O. basilicum* leaves extracts ameliorate cadmium-induced testicular damage in rats. The aim of this study was to assess the potential protective effects of aqueous extract of *O. basilicum* leaves in male rat model against diazinon- induced reproductive toxicity.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats of the inbred strain (*Rattus norvegicus*), weighting 150 ± 5 g were used in this work. Animals were obtained from the laboratory animal house of Egyptian Organization for Vaccine and Biologic Preparations, Helwan, Egypt. Rats were housed in polypropylene rodent cages, given water *ad libitum* and fed standard rodent pellet diet for 2 weeks for adaptation. Rats were exposed to a 12h light: 12h dark cycle, at a room temperature of 18-22°C. Maintenance of the animals was approved by the animal ethical committee in accordance with the guide for care and use of animals of Menoufia University, Egypt. The animals were divided into four groups.

Group 1: Control group (10 rats).

Group 2: Animals (10 rats) of this group were orally administrated aqueous *O. basilicum* extract at a dose level of 20 mg/kg 5 days / week for 6 weeks. Fresh leaves of *Ocimum basilicum* were collected from a garden within Faculty of Science, Menoufia University, Shebin El-kom, Egypt. The leaves were rinsed with clean water to remove any foreign matter. Leaves were dried in the shade and ground to a fine powder using a laboratory mixer. One hundred grams of leaf powder was refluxed with 750 ml of double distilled water for one hour and concentrated using rotary evaporator. The extract was kept at -20^{0}C until experimentation. The aqueous extract was used at a dose level of 20 mg/kg *O. basilicum.*[14]

Group3: Animals (10 rats) of this group were orally administrated diazinon at a dose of 20 mg/ kg.b.wt. (Hariri et al., 2010)[15] 5 days / week for 6 weeks.

Group 4:10 rats were given diazinon (20 mg/ kg b.wt) followed by oral administration with aqueous *O. basilicum* extract (20 mg/ kg) 5 days/ week for 6 weeks.

Animals from all represnted groups were taken for inspection and determination of biochemical parameters after 3 and 6 weeks.

Histological examination

The treated animals and their controls were anesthetized and dissected after 5 weeks of treatment. Testes were removed and fixed in 10% neutral formalin for 24 h, washed in running tap water for 12 h, and dehydrated in ascending grades of ethanol, cleared in two changes of xylene and embedded in paraffin wax and sections of 5 micrometer thickness were cut by rotary microtome. Slides were stained with haematoxylin and counterstained with eosin for histological examination. 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.

Immunohistochemical study

Immunohistochemical reaction was performed using an avidin biotin complex immunoperoxidase technique on paraffin sections. Formalin-fixed paraffin- embedded tissue sections were deparaffinized, endogenous peroxidase activity was blocked with H_2O_2 in methanol and the sections were heated in 0.01 mol/L citrate buffer in a microwave pressure cooker for 20 minutes. The slides were allowed to cool to room temperature and nonspecific binding was blocked with normal horse serum for 20 minutes at room temperature. The MIB-1 monoclonal antibody was used for detection of nuclear Ki-67, a marker of proliferating cells (code no: M7187, dilution 1:40, DAKO). Anti-Bax (Dako, Cambridge, UK) monoclonal antibodies were used for detection of bax. Counterstaining was performed using Mayer's hematoxylin (BioGenex, Cat. No.94585). For evaluation of each marker, the percentage of positively stained cells in the total number of cells under 40x magnification was calculated.

Biochemical study

At the end of the experimental period pieces of testes were quickly cut, weighed and stored at -20°C then 10%W/V homogenate was prepared by granding 0.3 g of tissue in 3 ml of saline. The homogenate was used to estimate oxidative stress parameters. Malondialdehyde (MDA) levels were determined as an indicator for lipid peroxidation.[16] Superoxide dismutase (SOD) activity was measured using the methods of Rest and Spitznagel (1977).[17] The principal of this method depends on the ability of SOD to inhibit the power of phenazine methosulphate mediated to reduce the nitro blue tetrazolium. Catalase activity was determined from the rate of decomposition of H_2O_2.[18]

Statistical analysis

All values were expressed as mean ± standard deviation (SD). The statistical analyses were carried out using SPSS statistical software (SPSS for windows, version 11.0). Differences between the groups were determined by one-way analysis of variance (ANOVA). Values were regarded as significantly different at $P < 0.05$.

RESULTS

Histopathology

Histological examination of testes of control rats showed normal testicular architecture with well organized seminiferous tubules. All stages of spermatogenesis from spermatogonia to mature spermatozoa and Sertoli cells were recognized in the tubules (Fig.1a). Testes of rats treated with basil revealed normal structure as control counterparts. Treating animals with diazinon revealed different histopathological alterations. After 3 weeks, intertubular hemorrhage was observed (Fig.1b). Large vacuoles were noticed in the germinal epithelium and degenerated cells were exfoliated in the center of the tubules (Fig.1c). After 6 weeks, the alterations were much more pronounced. Testes of these animals showed congestion of blood vessels (Fig.2a) and the seminifrous

tubules appeared devoid of spermatogenic cells except few spermatogonia (Fig.2b). Animals treated with Diazinon and ocemium showed that most of the seminiferous tubules appeared with large number of spermatogenic cells with an increase of sperms in comparison to 3 weeks- treated animals (Fig.2c).

Morphometry

Data in Figs 3 & 4 showed that treating animals with diazinon caused significant reduction ($P < 0.05$) both in the diameter of the seminiferous tubules and in their epithelial heights respectively. On the other hand, animals given diazinon and ocemium extract manifested an increase in the diameters of the tubules and the epithelial heights than diazinon treatment alone. However, no statistical significant difference was noted between control, ocemium and diazinon- ocemium treated rats.

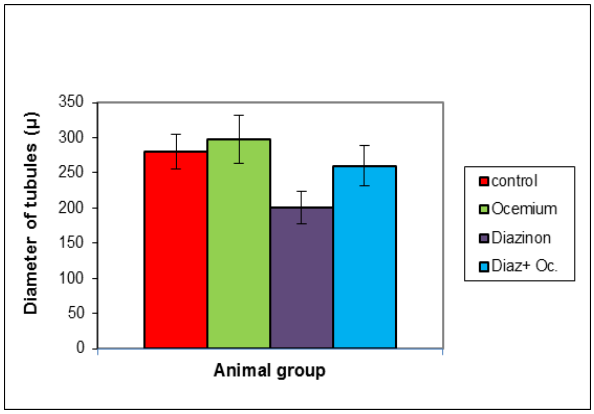


Fig. 3: showing the diameter of the seminiferous tubules. There was a significant decrease in tubule diameter in diazinon-treated rats' testes.

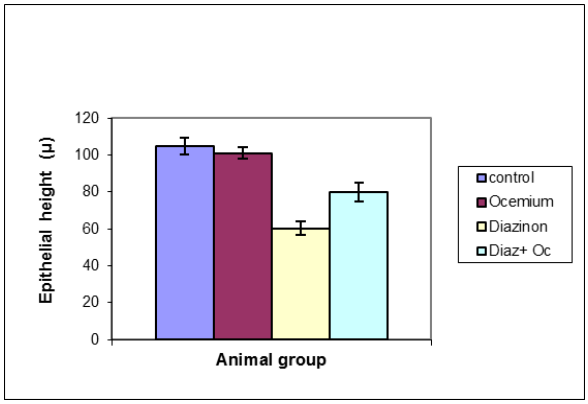


Fig. 4 showing the epithelial heights in the testis of all treated groups. It is obvious that diazinon-treated rats suffered the most deleterious action followed by diazinon- ocemium. No statistical significant differences were noticed between control and ocemium- exposed rats.

Immunohistochemical observations

Testicular tissue obtained from diazinon-treated rats for 6 weeks showed strong expression of bax in Leydig cells in comparison with the control group (Fig. 5 a and b). Treatment of animals with diazinon + ocemium extract decreased the expression of bax (Fig. 5c). In the seminiferous epithelium Ki-67 is expressed in the nuclei of spermatogonia. A normal expression of ki-67 was observed in control rats (Fig. 6a), whereas animals treated with diazinon revealed decreased expression of Ki-67 (Fig. 6b). Animals treated with diazinon + ocemium extract showed an increase in the expression of Ki-67compared to diazinon-administered rats (Fig.6c).

Biochemical results

Figure (7) showed that MDA increased significantly in diazinon – treated group in comparison with control group. Treating animals with diazinon and ocemium leads to decrease in MDA in the testicular tissue compared to diazinon alone. SOD and CAT activities were significantly decreased in rats treated with diazinon and their values increased after treatment with diazinon and ocemium (Figs. 8 & 9). No significant differences were recorded between control and ocemium groups.

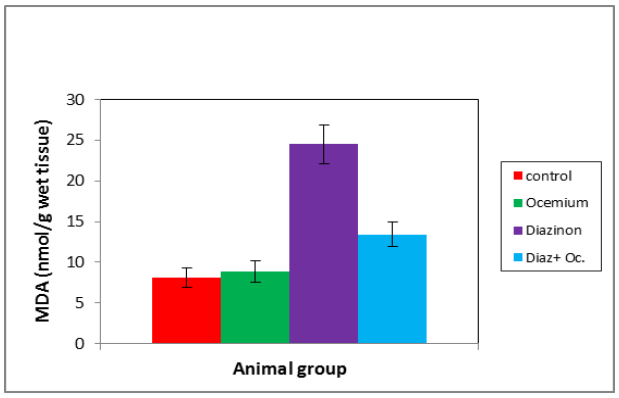


Fig.7: Malondialdehyde level in the testis of treated animals. Diazinon-treated rats showing the highest value and no statistical significant difference between control and ocemium. Treatment with ocemium decreased the level of MDA due to diazinon administration.

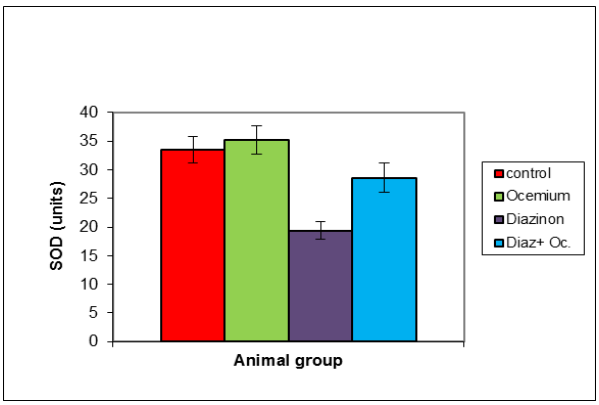


Fig.8 showing levels of superoxide dismutase (SOD) measured in the testes of rats subjected to different treatment. A significant depletion was induced by diazinon treatment compared to control or ocemium-alone treatment. However, administration of ocemium along with diazinon induced increase in SOD in comparison to diazinon-treatment alone.

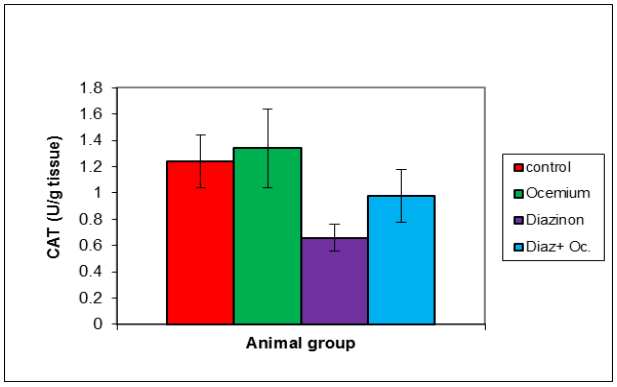


Fig. 9 showing the catalase (CAT) activity in the testes of rats. There was a significant decrease in the testes of rats treated with diazinon. However, ocemium administration with diazinon restored CAT activity to a large extent but still lower than that of control or ocemium alone treatment.

Fig. 1a: Section in the testis in a control rat showing the presence of normal seminiferous tubule with sperms in the centre (S). LC: Leydig cells. X400

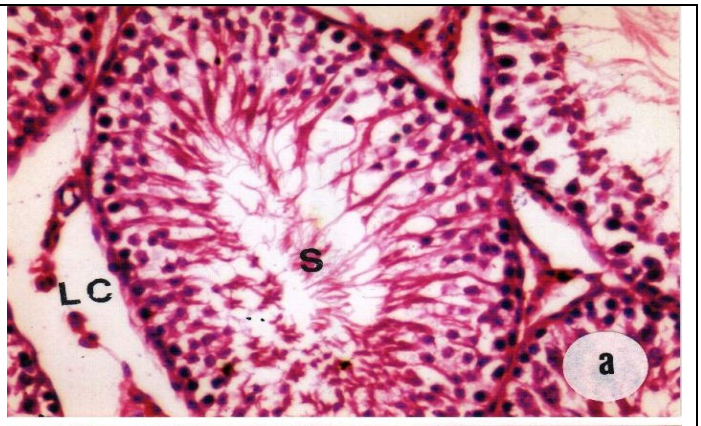


Fig. 1b. Section in the testis of a rat treated with diazinon for 3 weeks revealed hemorrhage in the intertubular CT (H) , shrinkage of the seminiferous tubule and desquamation of spermatogonia. X200

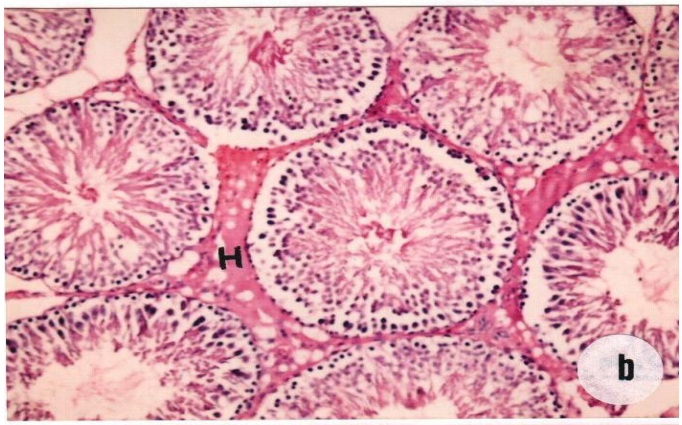


Fig. 1c: Section in the testis of another rat treated with diazinon for 3 weeks showing degeneration and exfoliation (E) of most spermatogenic cells, degeneration of sperms and the presence of vacuoles (V). X400

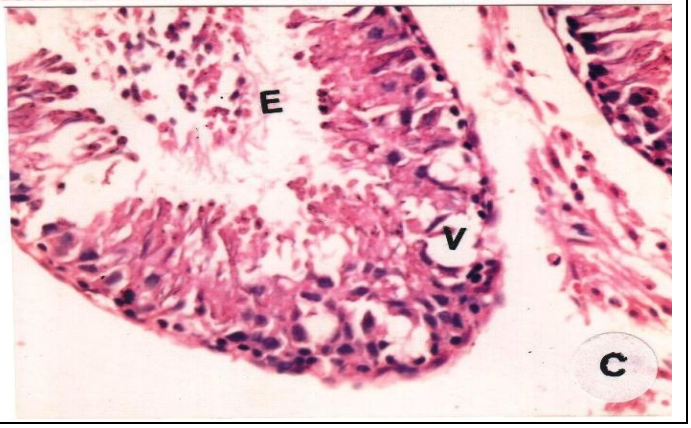


Fig. 2a: Section in the testis of a rat inspected following 6 weeks of diazinon administration exhibiting marked degree of histological abnormalities. Most sperms disappeared from the tubules, congestion of veins was evident (arrowhead), and degeneration of the intertubular CT. X200

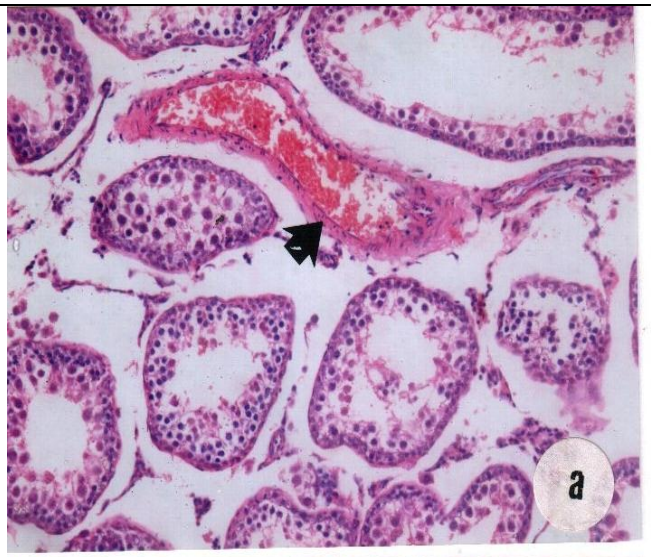


Fig.2b: Section in the testis of a second rat following 6 weeks of diazinon exposure showing a tubule almost devoid of sperms, condensation of spermatogenic cells nuclei and degeneration of intubular CT. X400

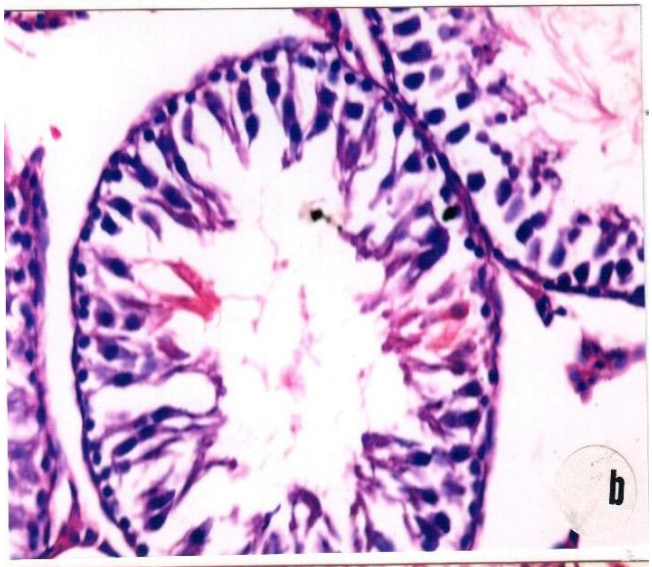
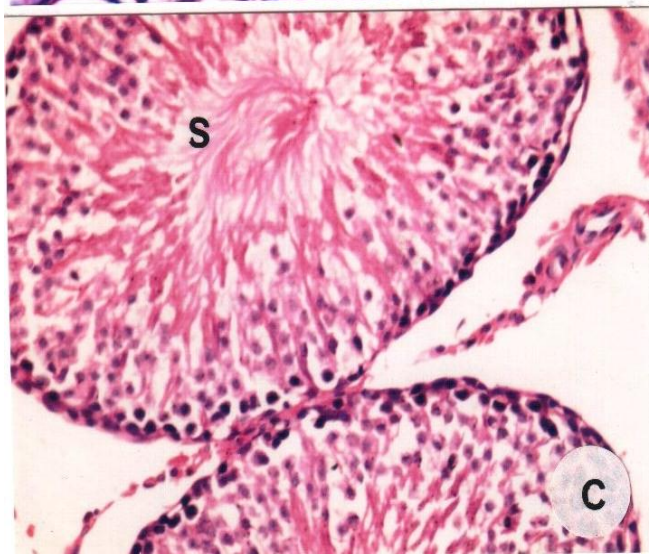


Fig. 2c: Section in testis of a rat exposed to the dual treatment i.e. diazinon and ocemium exhibited nearly normal histoarchitecture where normal numbers of sperms (S) and restoration of almost spermatogenic cells were observed. X400



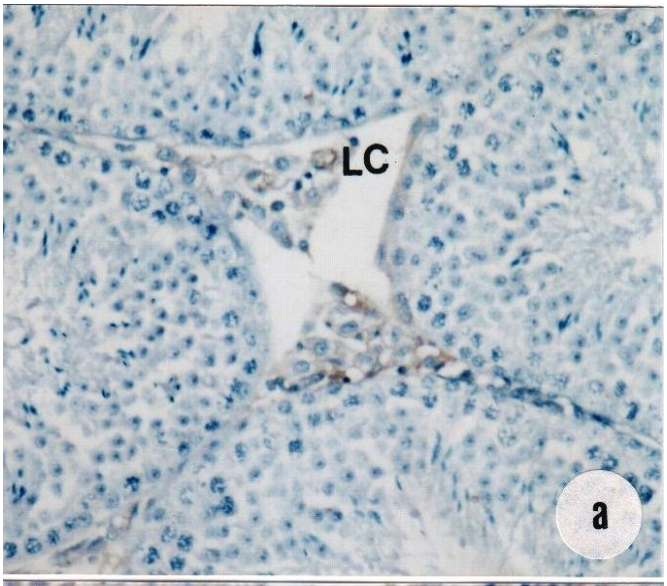


Fig. 5a. Bax expression in Leydig cells (LC) in the testis of a control rat showing moderate blue colour in the cells, immunostaining X400

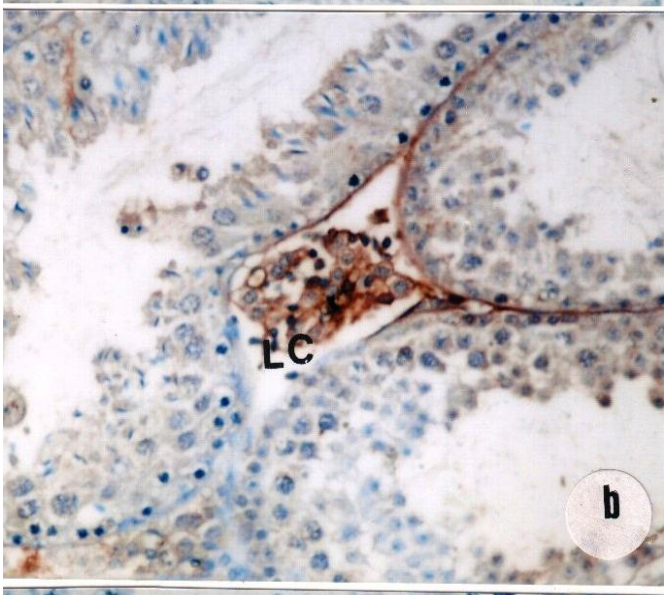


Fig. 5b: Strong bax expression in Leydig cells (LC) in the testis of a rat treated with diazinon for 6 weeks, immunostaining X400

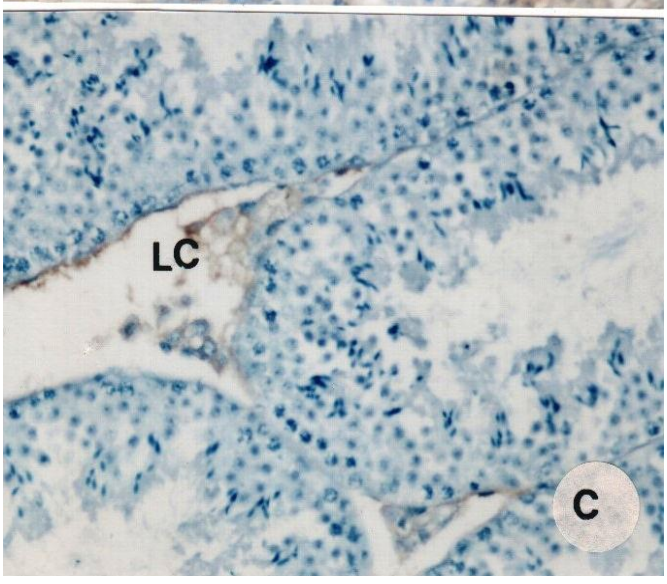


Fig.5c: Moderate bax expression in Leydig cells (LC) in the testis of a rat treated with diazinon and ocemium extract, immunostaining X400

Fig. 6a: A normal expression of Ki-67 in the nuclei of spermatogonia in the seminiferous tubules of a control rat, immunostaining X400

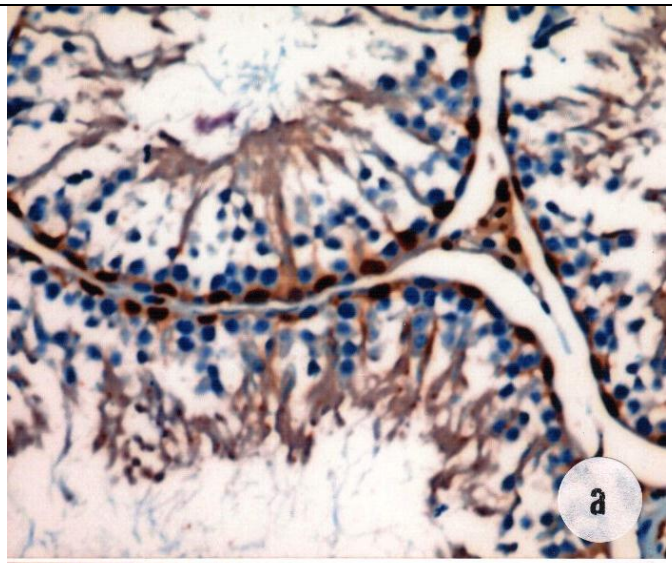


Fig. 6b.: Decreased expression of Ki-67 in the nuclei of spermatogonia of a diazinon- treated rat, immunostaining X400

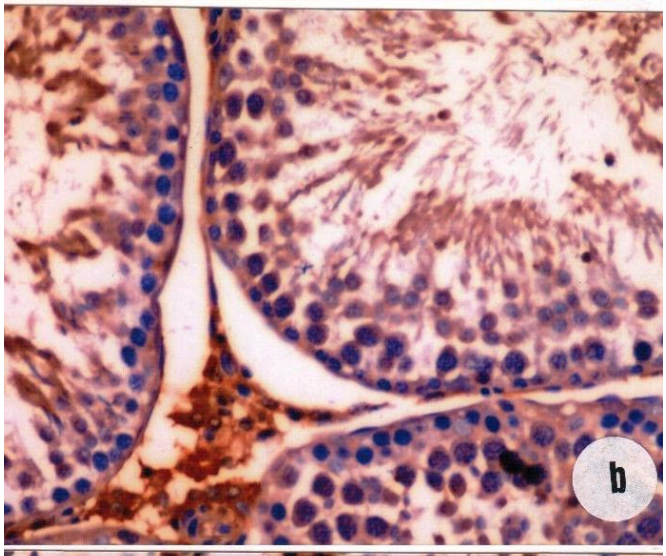
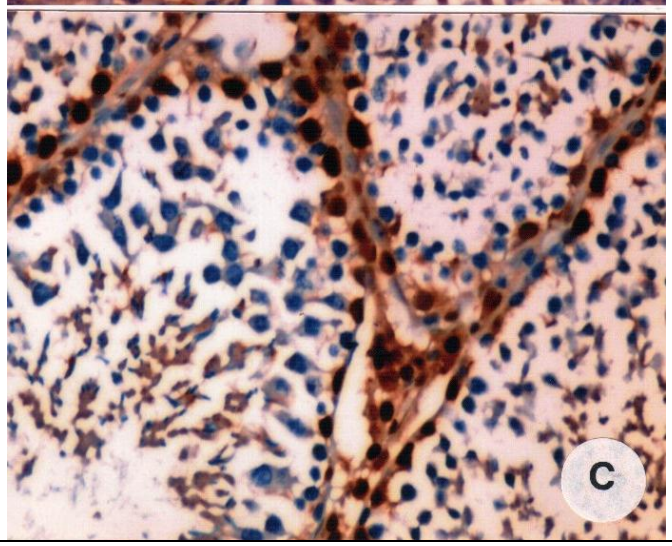


Fig.6c: Increased expression of Ki-67 in rats treated with diazinon+ ocemium extract compared to diazinon-administered rats, immunostaining X400



DISCUSSION

The present results revealed that diazinon exerted deleterious action on the testes of rats both histologically and biochemically. Many histological alterations were observed in the testis including degeneration of seminiferous tubules, intertubular blood hemorrhage as well as inhibition of spermatogenesis. In agreement with these results, Adamkovicova et al.[19] reported that diazinon caused a significant thickening of seminiferous epithelium, cellular degeneration, necrosis and vascular

dilation and congestion in the interstitial tissue of testis of rats. Other reports indicated that diazinon suppresses reproductive function with endogenous hormonal disruption[20] and caused spermatogenic disturbances.[21] Piña-Guzmán et al.[22] reported that exposing mice to diazinon negatively affects sperm motility and DNA integrity, which may contribute to reduced semen quality and concomitant decreases in fertility. A significant reduction was observed in diameter and weight of testes after diazinon (DZN) administration. Diazinon lead to significant reduction in sperm counts and spermatogenic, Leydig and Sertoli cells and a decrease in serum testosterone concentration.[23] Immunohistochemical results showed that diazinon decreased cell proliferation, as reflected by decrease of Ki-67 expression, and increased apoptosis as shown by increase of bax expression. In accordance with this result, Ali et al.[24] demonstrated that rats received DZN showed highly degenerated testes with remarkable atrophy and edema in seminiferous tubules and interstitial connective tissue as well. They suggested that the direct effects of the DZN induced sever apoptosis in the germinal cells and remarkable germinal cells degeneration lowered the sperm quality and quantity. Lari et al.[25] reported that diazinon caused increase of lipid peroxidation and induced apoptosis through activation of caspases-9 and -3 and increasing Bax/Bcl-2 ratio in rat liver. Other insecticides were found to induce apoptosis in testes of experimental animals. Sakr and shalaby[26] reported that that carbofuran induced testicular apoptosis as indicated by increase of caspase-3 and bax in germ cells.

Treating rats with diazinon caused decrease of SOD and CAT, while elevated lipid peroxidation. Anbarkeh et al.[27] reported that diazinon increased lipid peroxidation and reduced GSH level. Messarah et al.[28] showed that diazinon induces the production of oxidative stress by alteration of antioxidant enzyme activity and increasing lipid peroxidation. DZN at higher doses induces the production of free radicals and oxidative stress in rat tissues and strains by alteration of antioxidant enzyme activity, depletion of GSH, and increasing lipid peroxidation.[29] Increased oxidative stress in the testis is associated with the suppression of Leydig cell steroidogenesis disruption of spermatogenesis, and implications for male fertility.[30, 31]

Examination of testes of rats exposed to combined treatment with diazinon and ocemiun revealed marked improvement in the histopathological as as well as decrease apoptosis compared with those treated with diazinon alone. Similarly, Sakr and Nooh[13] repoted that O. *basilicum* extract alleviated cadmium-induced testicular damage and apoptosis in rats. Khaki et al.[32] reported that *O. basilicum* extract protected rats from testicular damage and reduced apoptosis after exposure to an electromagnetic field. Treatment of mice with NiCl2 exhibited significantly depleted GSH and led to apoptotic changes in the testis cells as evidenced by DNA fragmentation. The treatment with Nickel and Basil oil resulted in a significant improvement in all tested parameters.[33] Asuquo et al.[34] reported that *O. gratissimum* extract improved the testicular histopathological alterations in diabetic rats.

The present results also showed that treatment with *O. basilicum* extract reduced lipid peroxidation and increased the antioxidant enzymes, SOD and CAT. These results indicated that the *O. basilicum e*xtract can reduce reactive free radicals that might lessen oxidative damage to the testes and improve the activities of the antioxidant enzymes like SOD and CAT, protecting the testes from diazinon intoxication. These results are in agreement with other studies which proved the antioxidant effects of *O. basilicum* extract. Ramesh and Satakopan [35] reported that administration of *Ocimum sanctum* extract before and after cadmium intoxication resulted in a significant decrease in LPO levels and significant increase in SOD, CAT, GPx, GSH and ascorbate levels. The protective effect of methanol extract of *Ocimum gratissimum* and *Ocimum canum* on the oxidative stress induced by alcohol consumption in rats was studied by George and Chaturvedi.[36] Their results showed that a marked improvement occurred in the activities of CAT and SOD while LPO decreased in animals treated with the extract. Lee et al.[37] indicated that O. *gratissimum* leaf aqueous extract (OGAE) may be important in protecting H9c2 cells from H_2O_2-induced cell death by inhibiting the mitochondrial dependent apoptosis pathway. Kath and Gupta[38] showed that hydroalcoholic extract of *ocimum sanctum* leaves lowered levels of MDA and increased levels of SOD in animal models of peptic ulcer.

The therapeutic potential of O. *basilicum* rests on its contents of phenolic compounds. The main phenolic compounds in Basil are rosmarinic acid, lithospermic acid, vanillic acid, coumarinic acid, hydroksibenzo acid, syringic acid, ferulic acid, protocatheuic acid and caffeic acid[39, 40] and a wide array of other natural products including polyphenols such as flavonoids and anthocyanins.[41, 42] Thus, the protective effect of O. *basilicum* against diazinon toxicity could be the result of direct free radical scavenger and antioxidant properties.

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