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The pharmaceutical efferct of date palm fruit extract (*Phoenix dactylifera L.*) against amitraz-induced infertility in male rats

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Abstract: The present study was aimed to investigate the potential protective effect of aqueous extracts of the flesh of dates (*Phoenix dactylifera* L.) against amitraz-induced infertility in male rats. 100 male Sprague-Dawley rats (120-150 g) were used. The animals were randomly divided into 4 groups as follows: Group 1, control animals were given free access to food and water, group 2 received dates extract (1/10 w/v) , group 3 received 1/20 of LD_{50} amitraz and group 4 received dates extract and the same dose of amitraz as in group 3. Infertility was investigated by examining sperm count, viability, and motility. Estimated levels of testosterone, estradiol and histopathological analysis were also done. The present results showed significant decrease in sperms count, motility and viability in group III rats that received amitraz (P<0.001), increased percentage of sperm cell abnormalities and decreased serum estradiol and testosterone hormones to 49.8±3.2 and 1.5±0.20 ng/dL versus 56.7±2.4 and 3.3 ±0.37 ng/dL respectively compared with control. Histopathological examination of the testes also showed marked degeneration of spermatogenic cells associated with interstitial necrosis and blood vessel congestion. Spermatogenic cells and cellular debris were present in the seminiferous tubules lumen associated with moderate degeneration of spermatogenic cells lining of some seminiferous tubules with interstitial diffuse edema. The current results documented that date palm fruit extract possessed an anti-oxidant and anti-infertility effect. We concluded that amitraz-induced testis damage in rats can be ameliorated by administration of date palm fruit extract.

Keywords: Amitraz, dates palm, testis, testosterone, infertility.

INTRODUCTION

Amitraz, a formamidine insecticide and acaricide, was approved by the US Food and Drug Administration (FDA) and Environmental Protection Agency (EPA)(Jorens et al., 1997). This insecticide is widely used in fruit, cotton, and hops, and as a veterinary medicine for the treatment of ectoparasites such as ticks, mites, lice and other animal pests in pigs, cattle, sheep, goats, dogs and bees. Its pharmacological activities include monoamine oxidase inhibition (Moser and Macphail, 1985), α2-adrenergic agonist activity (Shin and Hsu, 1994), and inhibition of prostaglandin synthesis (Yim et al., 1978). Amitraz poisoning can occur through oral or dermal exposure and, potentially, by inhalation. At high doses of amitraz, the function of the hypothalamus can be reduced (Hypothermia et al., 2012). There are limited data on spermatogenesis, fertility, reproductive and developmental toxicity due to amitraz. Landmarks alterations due to amitraz exposure in rats were recorded (Palermo-Neto et al., 1994, Palermo-Neto et al., 1997).

Date palm (*Phoenix dactylifera*) is extensively planted in the hot and dry climate regions of Africa, the Middle East and Asia. Date palm fruit is an influential food resource in these regions. Besides food grade date production, large amounts of dates end up as a waste. The global waste palm date production is approximately two million tons/ year (Besbes et al., 2009). Palm date fruits consist of 3 main parts: date flesh, date pit, and skin. The glucose, fructose and sucrose are the main sugars of date flesh. The palm date fruit has a high content of sucrose at early stages of maturing, but during the maturation process it is converted to glucose and fructose. Proteins appear in date fruits as 1–3% of dry matter, while its fat content was reported to be 0.52–3.25% (Myhara et al., 1999). The information accrued in the past four decades suggest that dates possess diverse medical uses including anti-hyperlipidemic, anti-cancer, gastroprotective, hepatoprotective and nephroprotective activities and thereby serving as an important healthy food in the human diet (Baliga et al., 2011).

For several years, a special interest has been paid to oxidative stress; specifically, an excessive production of reactive oxygen species (free radicals). A large number of experimental and epidemiological studies have indicated that reactive oxygen species (ROS) cause to organ injury (Goetz and Luch, 2008). Reactive oxygen species are constantly formed as a byproduct of normal metabolic processes, and their generation is accelerated by accidental exposure to occupational chemicals like pesticides.

Although pesticides like amitraz may be valuable in agriculture, many pesticides or their breakdown products can be found in trace amounts or higher levels in air, soil and water. Environmental exposure to these agents may cause serious health risks which may include infertility. The objective of this study was to evaluate the anti-toxic potentials of date palm fruit extract (Phoenix dactylifera L.) on amitraz-induced infertility in male rats.

MATERIALS AND METHODS

Experimental animals

One hundred male Sprague Dawley rats weighing 120-150 g were obtained from the Animal House of the National Research Centre, Cairo, Egypt. The animals were maintained on standard laboratory diet and water *ad libitum*. After an acclimation period of one week, the animals were randomly divided into four groups and housed in cages at a temperature (23±1ºC), 40-60% relative humidity and artificially illuminated (12h dark/light cycle), room free from any source of chemical contamination. All animals received human care and use according to the guide lines for Animal Experiments which were approved by the Ethical Committee of Science Faculty, Fayoum University, Egypt. Experimental animals were divided into the following groups:

1) Control group; rats were fed on a standard diet and water *ad libtium* for two months.

2) Date palm fruit treated group; rats were fed on a standard diet , water *ad libtium* and received date palm fruit extract (10%/ kg) for two months.

3) Amitraz treated group; rats were fed on the same standard diet and given oral dose of amitraz at 1/20 LD50 for 2 months.

4) Amitarz treated and date palm fruit extract group; rats were fed on a standard diet and orally treated with amitraz and date palm fruit extracted for experimental period (Institoris et al., 2007).

Amitraz and palm date preparations

Amitraz

Liquid insecticide purchased from local company with concentration 12.5%. The dose of 1/20 LD50 (529 mg/kg b.wt), dissolved in corn oil was used (Institoris et al., 2007).

Date palm fruit extract preparation

Sakkoty date (dry) was from Egypt local market. Flesh was extracted two times with distilled water (1/10, w/v) by grinding with a mortar and pestle. It was centrifuged for 20 min at 3000 rpm and the supernatant was collected. We selected an aqueous extract because most of the antioxidant components in dates are extracted in water. During the experiment, the aqueous extract of Sakkoty date was daily prepared and administrated to rats (Vayalil, 2002).

Biochemical kits:

Enzyme immunoassay kits for estimating of the levels of serum testosterone and estradiol hormones in both control and treated rats were obtained from Gamma Group Company, Egypt.

Semen collection

After exposures to the various treatments, the animals were sacrificed by cervical dislocation. The abdomen was opened to harvest the right epididymis which was weighed and the caput epididymis lacerated on a glass slide in petri dish using a warm (27^0C) sterile lancet to release the semen sample.

Sperm Motility Study

Some drops of normal saline (at 27^oC) were added to the semen sample on the slide to potentiate full motility of the spermatozoa (Turner and Giles, 1982). The average gross motility was scored under the microscope x40 objectives (Oyeyemi et al., 2000). The motility scoring was carried out at room temperature (37^0C) to prevent heat or cold shock. The percent motility was determined by counting both motile and immotile spermatozoa per unit area. The testis were also harvested, weighed and stored in a normal saline.

Sperm viability test

Eosin-nigrosin staining was used to evaluate sperm viability (Björndahl et al., 2003). Briefly, eosin (1%) and nigrosin (10 %,) was prepared in distilled water. Two volume of sperm suspension was mixed with one volume of 1% eosin. After 30 second, nigrosin volume equal to eosin was added to this mixture. Thin smears were then prepared and left to dry on hot plate then observed under alight microscope at 100X magnification. Viable sperm remained colorless while nonviable sperm stained red. Two hundred sperms were counted for each sample and viability percentages were calculated

Sperm Count

Sperm counts were done under microscope using improved Neubauer hemocytometer. Counting was done in 5 large Thomas square and adjustment was made for the volume of the normal saline added.

The count was calculated from;

Counts / ml = No. of sperm cells in 5 large Thomas square X 32000 X dilution factor.

The epididymal fluid was diluted with normal saline by adding 0.9 ml to 0.1 ml of the crushed epididymis. The diluted solution was transferred into each chamber of improved Neubauer`s counting chamber

(hemcytometer) and sperm heads was manually counted under a light microscope. The ruled part was then focused and the number of spermatozoa counted in five 16-celled squares. The sperm concentration was then calculated and multiplied by 10^6 and expressed as (X) × 10^6/ml, where X is the number of sperm in a 16-celled square (Akang et al., 2010).

Histopathological studies:

Preparation of paraffin sections:

For histological preparations, animals were sacrificed and left testis was dissected out. It was cut into small pieces and immediately fixed in 10% neutral formalin for 24 hours. After fixation, specimens were dehydrated in ascending series of ethyl alcohol 70, 80, 90 and 96% for 30 minutes each, then in two changes of absolute ethyl alcohol for 30 minutes each. Tissues were cleared in xylol for 20 minutes (two changes) then impregnated in paraplast wax (three changes) at 60^{o}C for three hours and embedded in paraplast wax. Sections 4 to 5 µm thick were prepared using microtome and stained wiyh hematoxylin and eosin (An et al., 2003)**.**

Statistical analysis

Comparison was performed between the treated groups and untreated control using SPSS11windows program. The data were considered significant if *p* values were less than 0.05.

REULTS

Figure (1) showed a significant decrease in sperms count, sperm motility and viability in animals of group III (*P*<0.0001, 0.0001, 0.022 respectively) when compared with control.

The present study showed a significant changes in spermatocytes tail and multiple abnormalities in the animal group that treated with amitraz (P<0.05), the significant changes in spermatocytes tail abnormalities in animal group that oral injected with amitraz and treated with date palm extract were shown (P< 0.05), when compared with control animals. Concerning semen analysis it was found that amitraz decreased the sperm progressive motility, sperm count and viability, but increased the percentage of sperm cell abnormality as recorded in Table (1) and Figure (2). The most frequently seen sperm cell abnormalities in the seminal smears of amitraz injected rats were detached head and coiled tails and other types as demonstrated in Figure (3). Oral administration of palm date extract for two months to amitraz treated rats caused significant decrease in the percentage of sperm cell abnormality (P < 0.05).

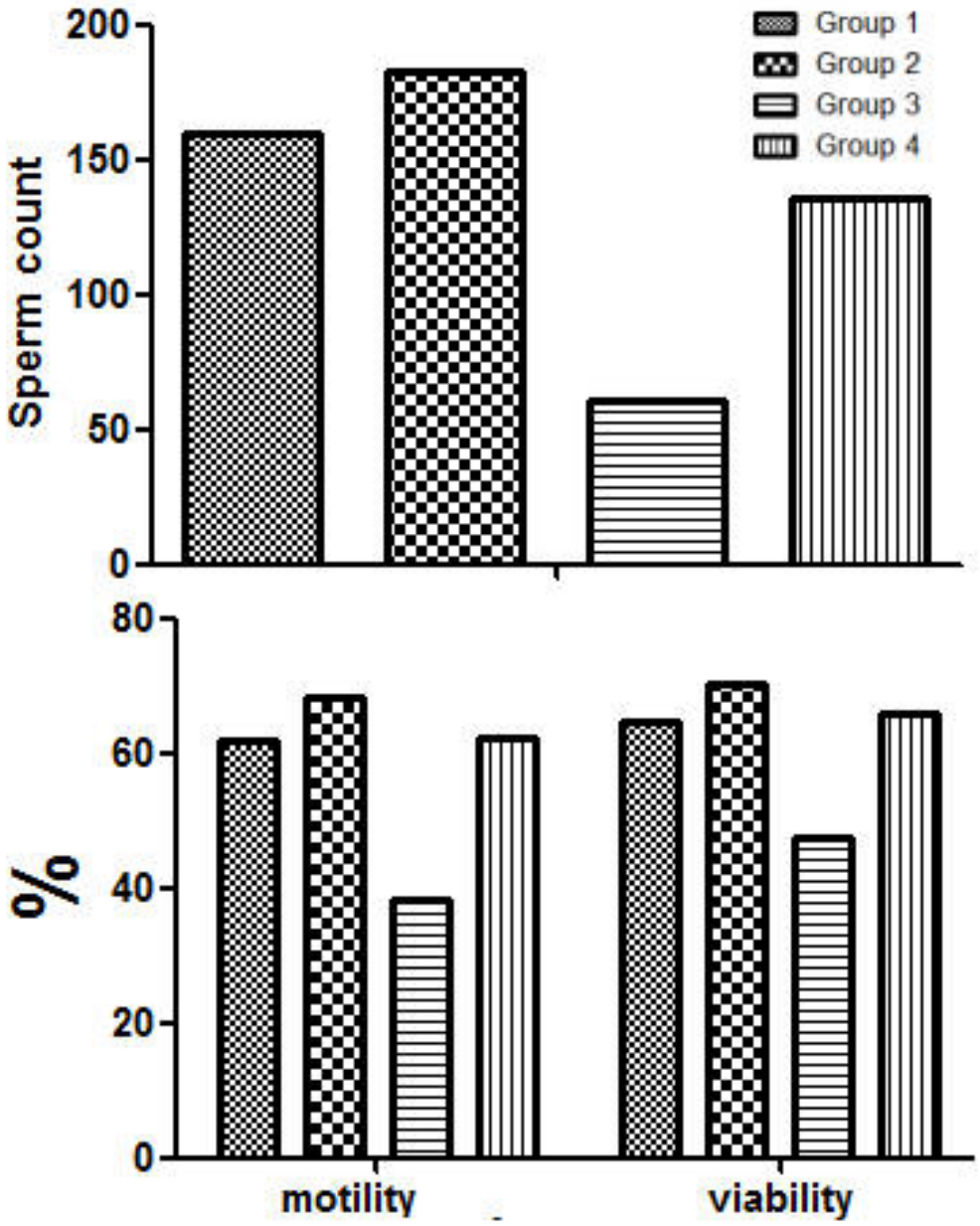

Figure (1): Spermatocytes count X 10^6 (Above), sperm motility and viability (lower) in different experimental animals groups.

Table 1: Showing the mean ± SD of sperm abnormalities in the different groups.

Groups	Sperm abnormalities%			
	Head	**Mid piece**	**Tail**	**Multiple**
GI	2.9 ±0.17	0.9 ±0.53	5.1 ±0.4	0.35 ±0.12
GII	0.9 ±0.51	1.9 ±1.34	6.3 ±1.53	0.12± 0.07
GIII	2.4 ±0.91	2.3 ±0.95	13.7 ±2.42*	3.09± 2.20*
GIV	1.9± 0.88	1.5± 1.24	10.1± 3.91*	1.9 ±0.90

$P \leq 0.05$ is considered significant, $P > 0.05$ is considered non-significant.

* Significant versus control.

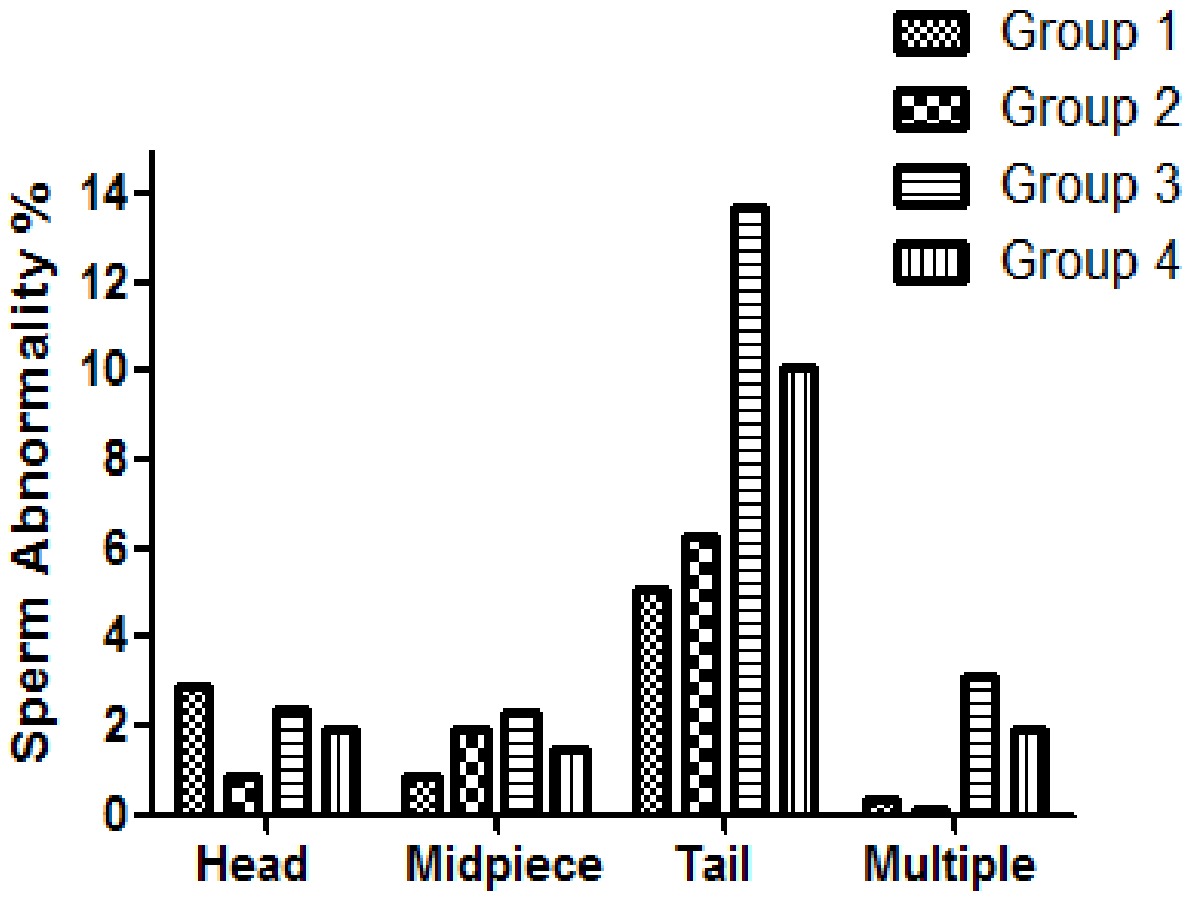

Fig. (2): Spermatocytes Abnormalities (Head, Mid-piece, Tail and multiple) in different experimental groups.

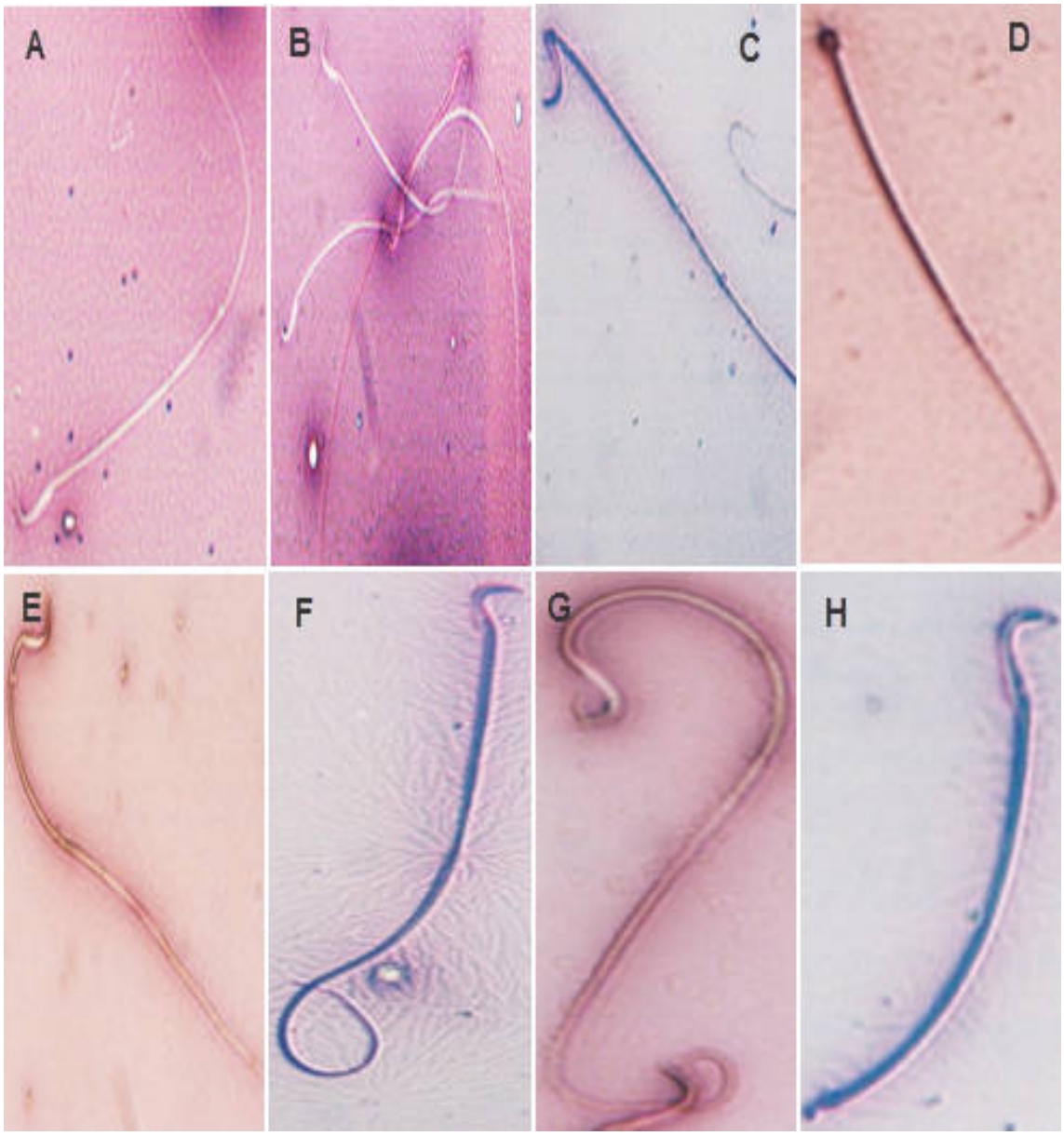

Fig. (3): Photomicrograph showing (**A**), the normal sperm morphology, (**B**) live spermatocytes (white color) and dead other sperms (red color), (**C**) abnormal sperms with bent neck, (**D**) abnormal sperms with pin head, (**E**) spermatocyte with curved mid-piece, (**F**) abnormal sperm with coiled tail, (**G**) spermatocyte with curved mid-piece and coiled tail and (**H**) spermatocyte with short tail and distal protoplasmic droplet.

Figure (4) showed that oral administration of amitraz to rats induced significant decrease in serum Estradiol and Testosterone hormones to 49.8$\pm$3.2 and 1.5±0.20 ng/dL versus to 56.7$\pm$2.4 and 3.3 ±0.37 ng/dL respectively ($P < 0.015$ and $P<0.00001$) in comparison to control rats. Oral administration of palm date fruit extract for two months to amitaz–induced rats caused increases in serum testosterone levels (3.5±0.77) as compared with the control rats (3.3 ±0.37).

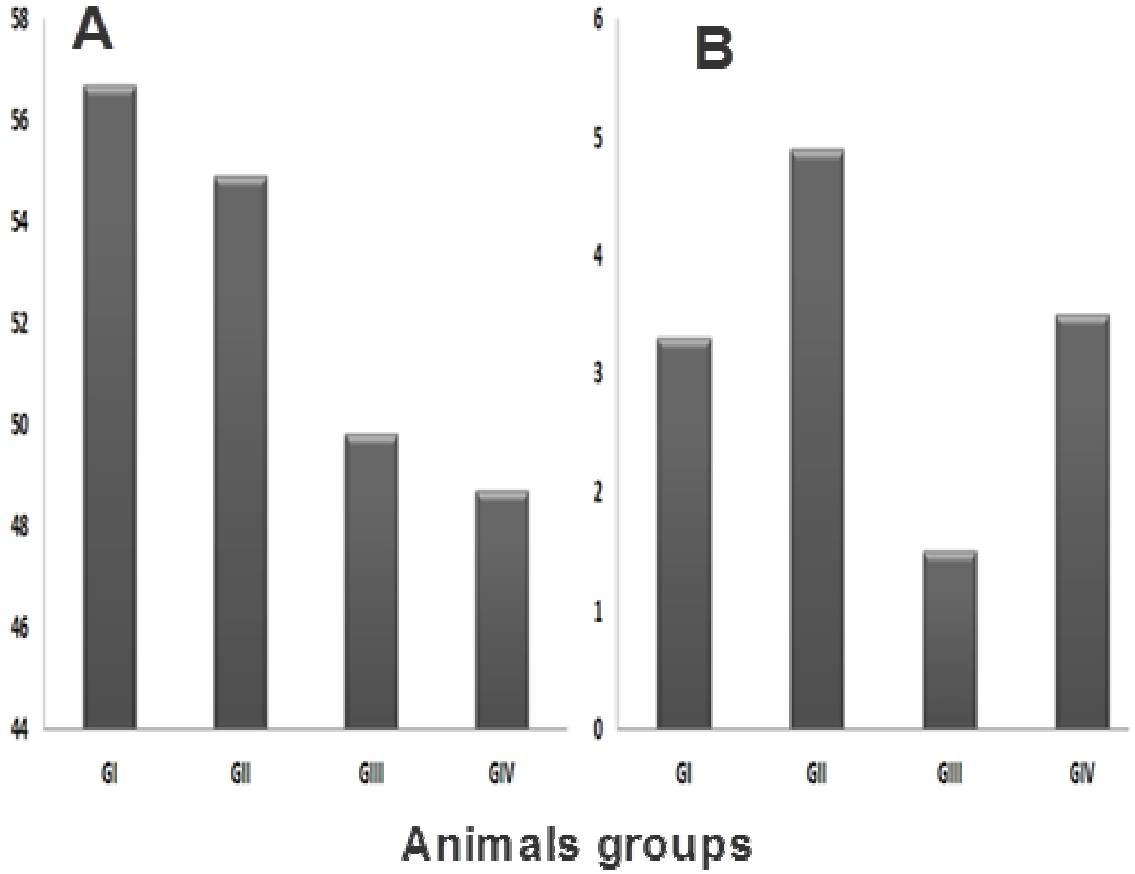

Fig. (4): The effects of treatment of amitraz rats with date palm extract on **hormonal concentrations (A): Estradiol and (B): Testosterone** for 8 weeks in the different experimental animals. All values are expressed as mean ± standard deviation.

Histopathological examination of the testes of normal rats revealed active mature functioning seminiferous tubules associated with complete spermatogenial cell series (Fig.5A). The testes of rats were given palm date extract alone showed normal histological structure of the seminiferous tubules (Fig.5B). The examined testes of rats given amitraz at 1/20 of the LD50 showed marked degeneration of spermatogenic cells associated with interstitial necrosis and blood congestion, also spermatogenic cells and cellular debris were present in the lumen of seminiferous tubules, testes of rats revealed moderate degeneration of spermatogenic cells lining some seminiferous tubules with interstitial diffuse edema (Fig. 5C, D & E). However, testes of rats given amitraz at 1/20 of the LD50 and palm date fruit extract showed an improvement of the histopathological lesions as revealed when compared with control animals, also, impaction of the lumen with spermatids and sperms which denoted complete spermatogenesis and normal arrangement of seminiferous tubule cells but only dilatation of seminiferous tubules was present (Fig.5F).

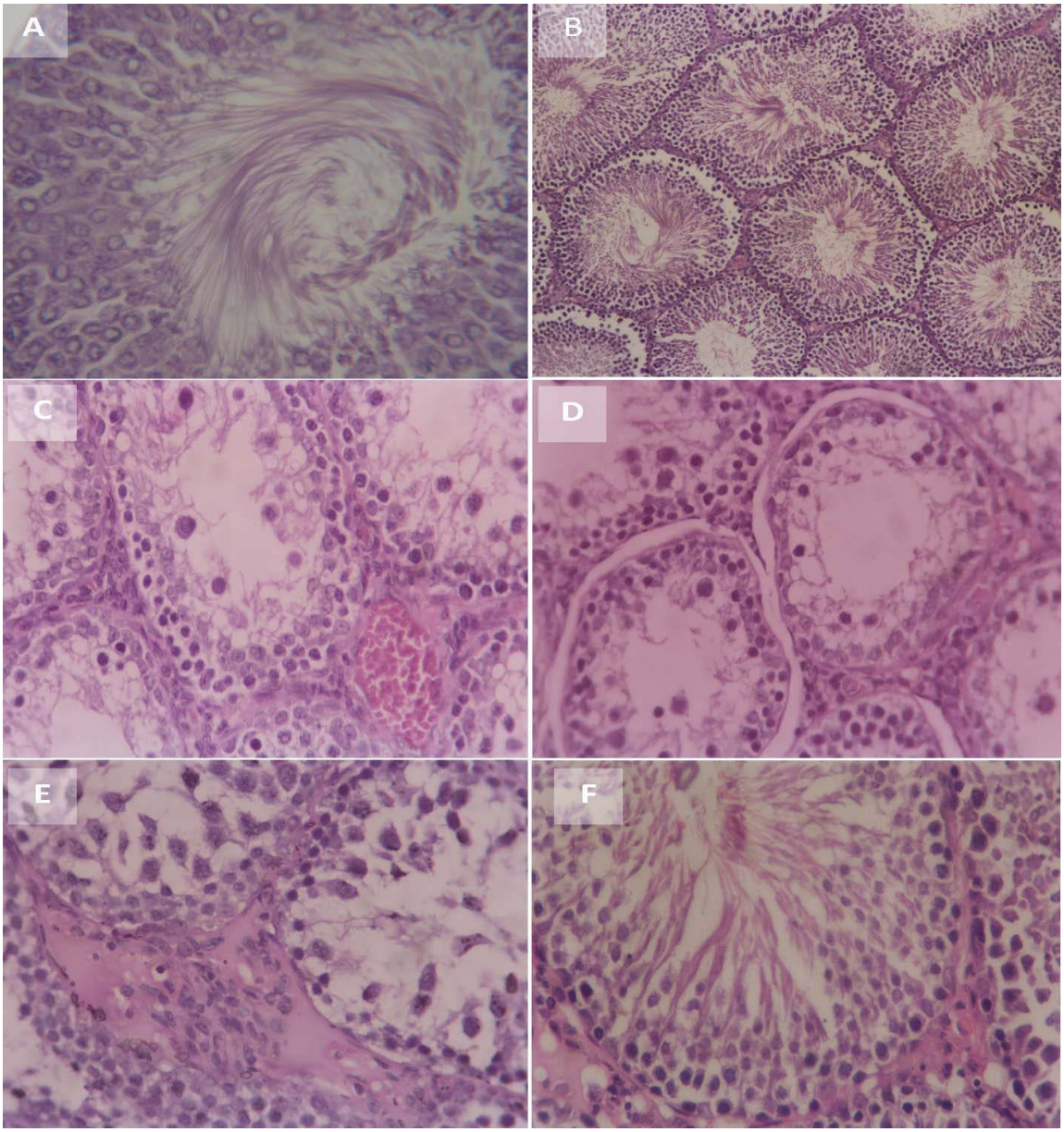

Fig. (5): Micrograph of transverse section of testis of (**A**) control rat showed normal seminiferous tubule. The seminiferous tubule has numerous sertoli cells, spermatogonium, and spermatocytes in the lumen of the tubule. (H&E x 200), (**B**) rat treated with date palm extract showed normal arrangement of seminiferous tubules which have numerous sertoli cells and different stages of the spermatocytes. (H&E x 100), (**C, D and E**) Testes of rats treated with amitaz acaricide showed destructed seminiferous tubules associated with degenerated spermatogonic cells, giant cells, vacuoles, congested blood vessel, absent interstitial leyding cells and free spermatocytes tubule lumeand free spermatocytes tubule lumen. (H&E x 400), (**F**) Testis of rat treated with amitaz acaricide and date palm fruit extract for 8 weeks showed normal arrangement of seminiferous tubule cells which have numerous cells sertoli spermatogonium, spermatocytes, with moderate hyaline regions and some vacuoles. (H&E x 400).

DISCUSSION

The present study was conducted to evaluate the adverse effects of amitraz pesticide on fertility and reproductive parameters of male rats. Amitraz pesticide was chosen because it is widely used in the Egypt as well as in many parts of the world. The animal model used in this study was used previously to assess the adverse effects of pesticides on fertility and reproductive parameters in laboratory animals (Costa et al., 1989). Animal studies have shown that oral feeding of date fruit increases the expression of antioxidant enzyme genes in rat cardiac tissue (Baliga et al., 2011). The observed antioxidant activity of dates has, been attributed to phenolic compounds, anthocyanins, flavonoid glycosides and procyanidins present in it and that sun-drying and ripening decrease the antioxidant activity (Amira et al., 2012). Selenium present in dates is also reported to contribute to the antioxidant effects. Multiple studies have shown that this essential trace element exerts its antioxidant function mainly in the form of selenocysteine residues that are an entire constituent of ROS-detoxifying selenoenzymes (thioredoxin reductases and possibly selenoprotein P (Steinbrenner and Sies, 2009). When considered in total it is very evident that the presence of diverse phenolic compounds (Aqil et al., 2006). To our knowledge, this is the first study to investigate the association between anti-infertility effects of date palm and amitraz acaricide toxicity .

Studies of sperm counts over time leave a critical question unanswered. It is important to know what could account for a precipitous decline in sperm production by otherwise healthy men. The environmental causes, especially those toxins that could affect human hormone systems (Carlsen et al., 1992). The results presented in this study clearly demonstrate that ingestion of amitraz for 8 weeks caused a significant decrease in the average sperm count in rats. This reduction in spermatocytes numbers is a clear indication of general reproductive toxicity. This extent of toxicity might have affected the animals indirectly rather than having any specific effect on fertility. Several reproductive parameters were adversely affected after ingestion of amitraz by adult male rats. So, exposure to pesticides lowers sperm levels below the restriction for male fertility.

In the present study, the sperm viability and motility were depressed with amitraz administration; where the abnormal sperm morphology due to elevated free radical oxygen species (ROS) production may serve as a useful indicator of potential damage to sperm DNA (Said et al., 2005). On the other hand, spermatozoa are highly susceptible to damage by extreme concentrations of ROS due to the high content of polyunsaturated fatty acids within their plasma membrane. Many studies have reported strong correlation between fertility and sperm quality (Duty et al., 2004). Sperm count, sperm motility, and viability significantly decreased, and several morphologically abnormal sperms were observed in rats that received amitraz. This indicates the inhibitory effect of amitraz on programming of spermatogenesis, thereby resulting in less sperm density. Impaired sperm motility may also result in infertility because of failure of sperm to reach the site of fertilization as well as their ability to penetrate through the zona pellucida of the ovum.

Our results showed that oral administration of amitraz to rats for two months significantly decreased testosterone and estradiol levels, but administration of dates extract restored testosterone level back to control. These results are in agree with the study that reported as exposure of male rats to amitraz decrease circulating testosterone concentration which dramatically suppress the stimulated release *in vitro* of GnRH from hypothalamic tissue (Goldman et al., 1999).

The prsesnt results contradict with other study that reported the administration of amitraz intraperitoneally to male rats for 4 days increases serum testosterone concentrations (Chou et al., 2008). Our results are also in agreement with the study that reported the dates fruit extract have been shown to increase sperm count in guinea pigs and to enhance spermatogenesis and increase the concentration of testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) in male rats. The formamidine pesticide (amitraz and chlordimeform) interfere with reproductive function because they disrupt the neuroendocrine regulation of gonadal function (Goldman et al., 1999).

The restored testosterone level to the normal level may be due to the fact that the date palm fruit extract had neuroprotective character, this is in agreement with the study that reported the Aqueous Date Fruit Extract (ADFE) has neuroprotective effect due to its anti-oxidant property (Majid et al., 2008).

Extract from date palm fruit is useful in controlling blood cholesterol level and protection of neurons against oxidative injury (Panahi and Asadi, 2009). Flavonoids are known to possess anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, anti-thrombotic, neuroprotective, and anti-carcinogenic activities (Sala et al., 2003). The present study suggested that amitraz affected normal male fertility due to reduction in testosterone production, this results are in agreement with the study that reported the increased Thiobarbituric Acid Reactive Substances (TBARS) and decreased sperm motility attributed partly to concomitant reductions in semen fructose and/or testosterone production where fructose formation by the accessory glands is dependent upon testosterone secreted by the testis (Al-Thani et al., 2003). The results suggest that sexual and aggressive behaviour are very susceptible to the toxicity produced by amitraz and could be explained by the direct or indirect effect of it on the testes and the influence on androgen biosynthesis. As testosterone plays a key role in sexual reusability as well as cause of aggressive behaviour in males and this in turn is equated with violence (Stanbrough et al., 2006). Our data presented in this work strongly indicates a serious decrease in the level of testosterone in the amiratz-injected group . This might be due to the degenerative testicular changes exerts in the testicular tissue as showed in histopathological examination in the present study.

Our histopathlogical examination revealed that the examined testes of the treated rats with amitraz showed marked degeneration, vacuolization of seminiferous epithelium, necrosis of germ cells, interstitial hemorrhage and necrosis of leydig cells. There is paucity of information on the effect of amitraz on the lipids of the testes. As regards to the histopathological results, the testicular degenerative changes induced by amitraz in intoxicated rats, as demonstrated in this study, agree with those of the study that reported the variable degrees of degenerative changes in the seminiferous tubules up to total cellular destruction after chronic exposure of male rats to insecticide methomyl(Mahgoub and El-Medany, 2001). The higher doses of pesticides for example carbofuran, carbamate insecticide, induced moderate edema, congestion, damage to Sertoli cells and germ cells, along with the accumulation of cellular debris in the lumen of a few seminiferous tubules which showed disturbed spermatogenesis. The testicular damage induced by amitraz in this study confirms the reported lowered fertilizing capacity of the treated rats. The toxic effect of amitraz on male reproductive system of the rat could be possibly explained by its direct cytotoxic effect and/or indirectly via decreased serum testosterone level. Combination of date palm fruit extract with amitraz significantly antagonized its reproductive toxicity in the treated rats (Pant et al., 1995).

Of greater importance to the public is the effect of ingesting normal *ad libitum* levels of date palm, especially because it is an inexpensive and effective prophylactic and/or treatment against amitraz induced infertility and a dynamic male fertility support. This study, along with other research, targets *Phoenix dactylifera* L. as a potentially secure and effective plant that has important medicinal values and benefits. Also, the present study has demonstrated that, date palm fruit extract possess an anti-infertility against amitraz acaricide activity and have a useful effects on spermatogenesis and sperm parameters in rats.

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