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Histopathology of male *Swiss albino* mice reproductive system due to toxic effects of thiamethoxam

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Abstract
The present study seeks to investigate the effect of thiamethoxam on the histology and physiology of male mice reproductive system. This study was conducted at College of Veterinary Medicine-Baghdad, from August to December 2016. Fifty adult males Swiss Albino mice, aged two months, were divided into five equal groups. The first group was immunized with *B. abortus* antigen. The second group was given thiamethoxam orally. The third group was treated with the same treatments in group 1 and 2. Group 4 was injected with virulent *B. abortus.* Group 5 acted as negative controls. All animals were euthanized and blood samples were taken for hormonal analysis. Pieces of testes and epididymis were put in 10% normal buffer formalin for routine histopathological sectioning. Results showed that thiamethoxam significantly decreased the sperm viability, motility and testosterone levels. In conclusion, thiamethoxam has a toxic effect for spermatogenesis in mice. The use of thiamethoxam should be experienced to avoid any harm to human and livestock.

Keywords: Histopathology, thiamethoxam, testesterone, testis

Abbreviations: CFBAgs: Culture filtrated *B abortus* antigen; nAChRs: nicotinic acetylcholine receptors; IP: Intraperitoneal; ROS: Reactive oxygen species; TMX: Thiamethoxam

1. Introduction
Thiamethoxam (TMX), a relatively new pesticide, belongs to second-generation of neonicotinoid pesticides, a group of several different insecticides, with mostly applicable, imidacloprid: thiacloprid and clothianidin [1]. Pesticides are classified according to their target, their mode or period of action, or their chemistry [2]. The wide use of TMX is attributed to its efficacy at low doses, variety of application methods, and long half-life [3]. It is bio-activated by cytochrome P450 enzymes via desulphuration to its related Oxon derivative [4]. There is a close resemblance of TMX chemical structure to nicotine: rendering it as agonist in mammals [5].

Although TMX is believed to be less toxic to mammals due to its lower interaction with vertebrate nAChRs compared to insect's [6], ingestion of a large amount could develop high toxicity targeted at the reproductive system [7]. Effects on male fertility can directly damage spermatozoa, alter Sertoli cell or Leydig cell function, or interfere with endocrine function in any stage of hormone control (hormone synthesis, release, storage, transport, and removal; receptor recognition and binding), thyroid function and the central nervous system [8]. Chemical changes in sperm nuclear proteins during the final stages of spermatogenesis are part of the causes of male reproductive toxins [9].

The insecticides used in commercial construction can be toxic and dangerous to a growing fetus [10]. They tend to alter human development, usually because they have endocrine agonist or antagonist activities and alter hormonal and genetic regulation [11]. In many epidemiological studies, declining sperm quality has been reported in agricultural workers [12]. In animal models, the results showed that a single dose of diazinon (8.125 mg/Kg, IP) given to mice could alter sperm function, mobility, morphology and ability to conceive [12].

In vitro, TMX induces lipid peroxidation, alters the functions of antioxidant enzymes and DNA damage [13]. Sublethal doses can lead to reduced growth and propagation of aquatic invertebrate, disruption of algae-invertebrate interactions, reduced egg production, delayed sexual maturation, changes in mammal behavior and disruption of ovarian structure [14].

Other studies have shown that exposure to natural chemicals such as diazinon and malathion causes cancer or reduces reproductive functions in animals and humans [15]. The present study is designed to investigate the effects of TMX on the level of sex hormones and histopathology of testis in adult male mice .

2. Materials and Methods

2.1. Chemicals, bacterial isolate and experimental mice

Thiamethoxam (Sigma, St. Louis, MO) and other chemicals were of standard analytical grade. The virulent culture of *B. abortus* was obtained from Dept of Microbiology, College of Veterinary Medicine-University of Baghdad. Fifty, 2 months old, adult males Swiss Albino mice at the age of two months were purchased from the Institute of Vaccine and Sera, Ministry of Health, Baghdad. Mice were kept in plastic cages for 5 days at the Animal House to adapt to the conditions before the start of the study. Pellets and water are given per ad libitum. All tests were performed at the Lab of Pathology, College of Veterinary Medicine-University of Baghdad, from August to December 2016.

2.2. Experimental design

All Experimental procedures were approved by the Animal Care and Use Committee (approval no. 1689/23 Aug 2016). Mice were divided into five equal groups, 10 each. Group 1 (G1) consists of mice immunized with CFBAgs (protein concentration 4.2mg/ml), two IP doses at two weeks intervals. Group 2 (G2) were daily gavaged with TMX (83.7mg/Kg BW). Mice of group 3 (G3) were administered both treatments of G1 and G2 for 9 weeks, and IP injected with 0.3 ml of live virulent *B. abortus* suspension containing 1 x 10^9 CFU/ ml. Mice in group 4 (G4) were IP injected with same dose and concentration of the same bacteria, one month after the experiment started and served as positive control. The 5th group (G5) was orally administrated with 0.3 ml of normal saline and served as negative control.

2.3. The Median lethal dose (LD_{50}) of thiomethoxam

The "Up-and-down" method [16] was used to determine the lethal dose (LD50) of TMX. Six albino mice weighing (20–25) gm were given 750-850 mg TMX (40mg/ml DW, orally) per Kg bodyweight. The dose difference was 50mg/Kg BW. The LD50 was calculated after observation the lethality in the dosed animals within 24 hours. The LD50 is calculated by using the equation

$$LD50 = xf + kd$$

xf =The final dose given
k = factor value from appendix
d = difference between dosing levels

2.4. Assay

The filtered culture of *B. abortus* antigen (CFBAgs), prepared according to Quin *et al*. [17], was used for vaccinating animals. Hormonal assay was performed by radioimmunoassay as described by Schulster *et al*. [18]. Abnormal Sperm Assay: Spermatogenesis in mice was performed according to Bruce and Heddle [19]. The caput and cauda epididymis extracted from male mice were placed in a petridish containing 1ml of physiological saline and then minced and teased carefully with fine scissors and forceps to release the spermatozoa. After gentle pipetting, the suspension is separated from the tissue fragments and to this suspension, a drop of 1% Eosin Y solution (10:1) was added for 30 minutes. Air-dried smears were prepared on clean grease-free glass slides using another clean slide angularly positioned at 300 to spread the drop through the whole length of the slide. The slides were then coded and cytologically examined under 40x binocular light microscopy. Eight separate slides were prepared for each mouse randomly. Percentage of active sperms was estimated as in the following equation:

Viability= [Number of live sperms/Total Number of sperm] x 100

Motility= [Number of motile sperms/Total Number of sperm] x 100

2.5. Histopathology

Pieces (o.5 cm3) of testes and epididymis were prepared and fixed in 10% normal buffer formalin for 36-48 hours and processed for routine histopathological examination [20].

2.6. Statistical analysis

Data was analyzed by one way ANOVA using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). The significance level was selected at $P<0.05$.

3. Results

Significant reductions in sperm count and spermatogenesis, as well as a decrease in serum testosterone concentration were observed (Table, 1). Testes showed micro-lesions represented as degenerative changes in seminiferous tubules, Leydig and Sertoli cells compared to the control groups. Seminiferous tubules of testes of Mice in G1 showed normal stage of spermatogenesis (Fig. 1). Epididymis filled with sperm (Fig. 2). Mice in G2 showed that inflammatory cells mainly neutrophil infiltrated in sub epithelial layer of the epididymis and in the interstitial tissue (Fig. 3) as well as focal hyperplasia of the epithelial lining cells of the epididymis. In addition, there were few or no sperm in the epididymis and incomplete mature spermatogenesis. Homogeneous material and cellular debris were observed in the lumen (Fig. 4). The main lesion in the testes of mice in G3 characterized by few or no sperm in the seminiferous tubules with round multi nuclei cell in the lumen (Fig. 5) accompanied by homogeneous material and cellular debris (Fig. 6). Mice in G4 (positive control group) showed lesion characterized by few or no sperm in the epididymis (Fig. 7) and incomplete mature spermatogenesis (Fig. 8). Vice versa, there was no significant microscopic findings in G5 (negative control) mice.

Table 1: Testosterone concentration, sperm motility and viability in thiamethoxam treated mice at 9 weeks post–treatment

G	G1	G2	G3	G4	G5
Testosterone (ng/ml)	77.8±0.3^{a}	19.7±0.22^{f}	55.1±0.21^{d}	60.0±0.57^{b}	54.24±0.18^{e}
Sperm motility (%)	32.0±2.3^{a}	25.0±0.69^{c}	28.0±0.5^{b}	20.0±0.3^{d}	^{a} 30.0±2.3
Sperm viability (%)	38.2±0.5^{a}	37.0±0.6^{e}	5.0±0.3^{c}	3.0±0.8^{d}	40.1±1.2^{b}

Data expressed as mean ± SE.
Different superscript within rows refer to significant differences at $p<0.05$.

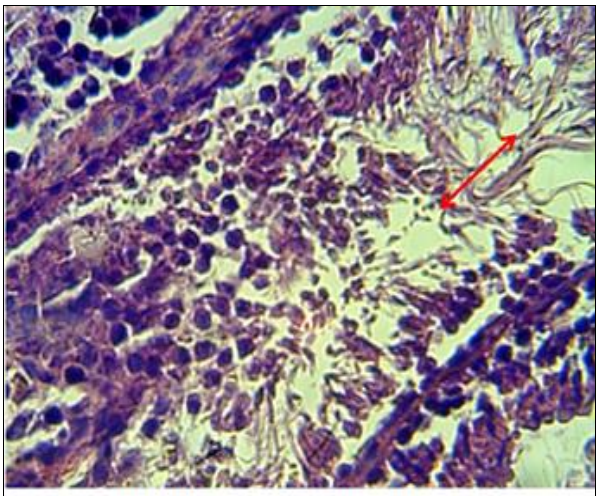

Fig 1: Histomicrograph in the testis of mice immunized with CFBAgs, filled with sperms (red arrow) (H&E stain, 40X)

Fig 2: Histomicrograph in the epididymis of mice immunized with CFBAgs. Note the normal stage of spermatogenesis (red arrow) (H&E stain, 40X)

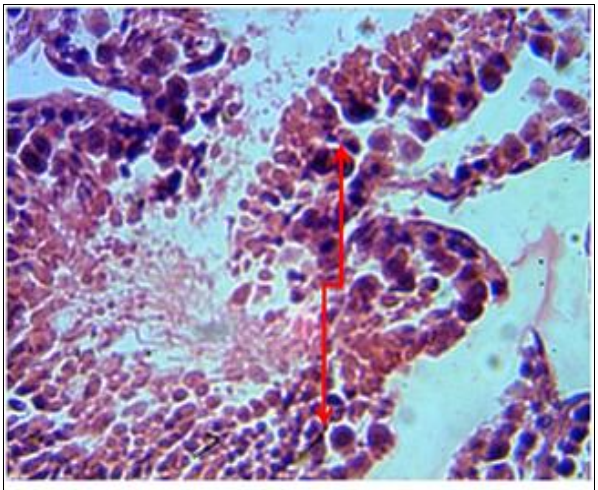

Fig 3: Histomicrograph in the epididymis of mice showed the effect of daily administration of thiamethoxam. Note the infiltration of neutrophil (red arrow) in sub epithelial layer and in the interstitial tissue (H&E stain, 40X)

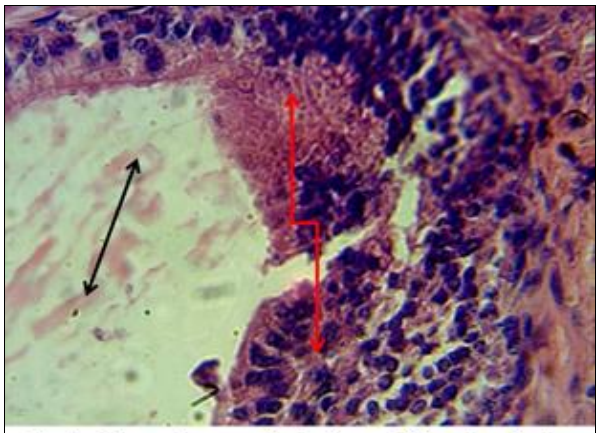

Fig 4: Histomicrograph in the epididymis of mice showed the effect of daily administration of thiamethoxam. Note the focal hyperplasia (red arrow) of the epithelial lining cells and scarce sperms with immature spermatogenesis (black arrow) (H&E stain, 40X)

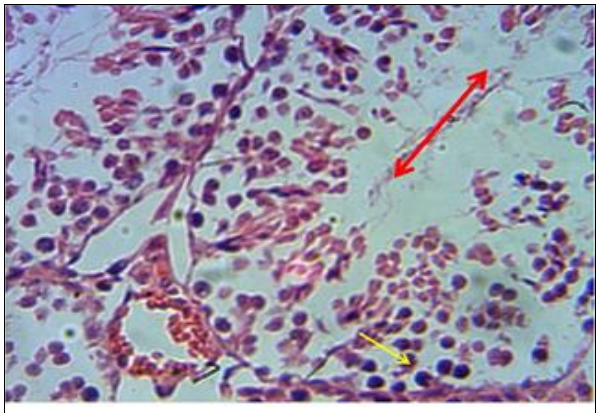

Fig 5: Histomicrograph in the testis of mice immunized with CFBAgs and daily administration of Thiamethoxam. Note, few or no sperm (red arrow), with round multiple nuclei cell (yellow arrow) in the lumen (H&E stain, 40X)

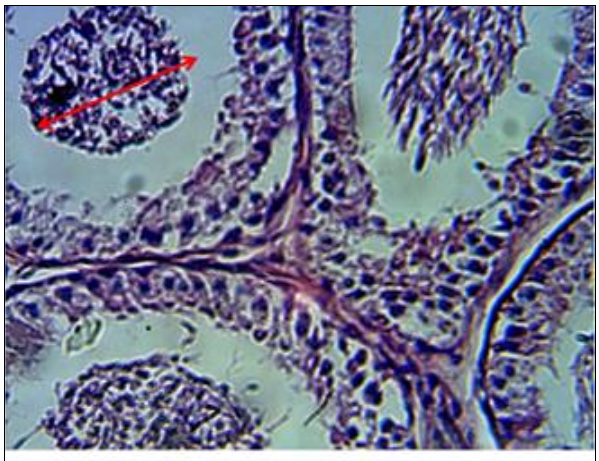

Fig 6: Histomicrograph in the testis of mice immunized with CFBAgs and daily administratic of Thiamethoxam. Note the few homogeneous material and cell debris (red arrow) in the lumen (H&E stain, 40X)

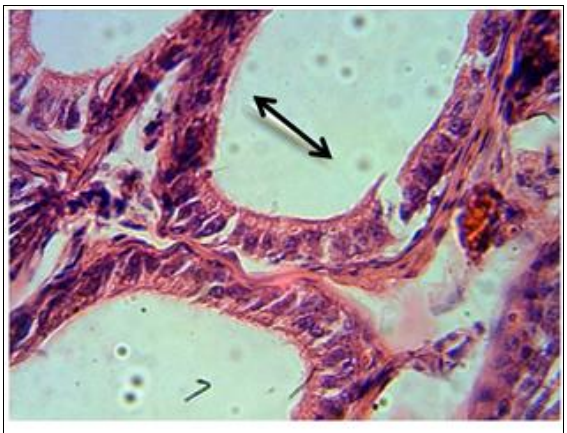

Fig 7: Histomicrograph in the epididymis of mice injected I/P with 0.3 ml of bacterial suspension (1 x 10^9 CFU/ ml) of live virulent *B. abortus* showing few or no sperm (black arrow) in epididymis (H&E stain, 40X)

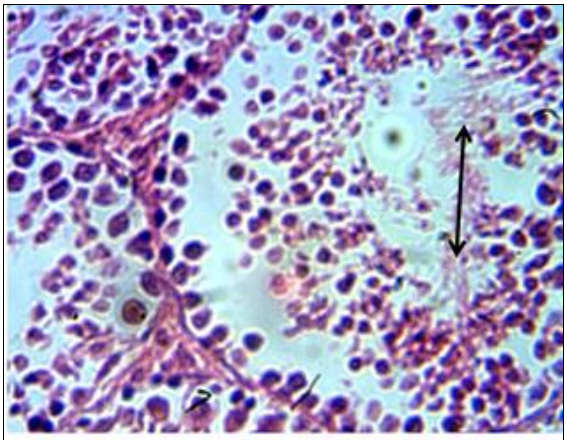

Fig 8: Histomicrograph in the testis of mice injected I/P with 0.3 ml of bacterial suspension (1 x 10^9 CFU/ ml) of live virulent *B. abortus* showing immature spermatogenesis (black arrow) (H&E stain, 40X)

4. Discussion

The present study showed a significant decrement ($P \leq 0.05$) in serum testosterone levels of TMX-treated mice compared with other groups, particularly the negative control group. This refers to that TMX induced oxidative stress mediated free radical production, damaging the testicular cells responsible for Testosterone production. Coinciding the present study result, El-Shenawy *et al*. [21] argued that pesticide is incriminated in increasing ROS or malondialdehyde and decreased the level of the antioxidants GSH, GSH-Px, SOD or catalase resulting in marked oxidative stress. Testosterone is necessary for sex organs differentiation, spermatogenesis and male fertility [22]. Prolonged exposure to toxic pesticides leads to hormonal disruption leading to fertility, adrenal exhaustions [23] and decreased serum steroid hormone levels by increasing catabolism and elimination of steroids [24]. Eventually, diminished serum level of testosterone is due to the effects of TMX causes decline in spermatogenesis and fertility in male. Moreover, this study showed a significant decrement ($P \leq 0.05$) in sperm concentration and motility which is mainly affected by serum Testosterone in mice treated with TMX as comparable with their levels in other groups. This evidence is coincided with Maxwell and his colleague [25] who revealed that acetamiprid significantly reduced the weight of whole body, testes, seminal vesicle, epididymis and prostate. Moreover, acetamiprid significantly decreased serum testosterone concentration, as well as sperm count, performance, viability and motility.

Sperm count is highly correlated with malformations and histological deteriorations, both of which are fundamental criteria of early testicular toxicity due to chemicals [26]. Abnormalities may be caused by genes in the sperm head or perhaps due to an abnormal chromosome complement. Severe pathological lesions in the testes of mice treated with TMX, as presented by our study, may be due the generation of ROS, which cause severe pathological changes in the reproductive tracts. This idea is consistent with Dutta and Meijer [27] who reported immature histological appearance of the testis, manifested by marked reductions in spermatogenesis, moderate tubular atrophy and increased spermatic giant cells in the testes of all TMX treated mice. Lack of testosterone level, which is requested along with Sertoli cells to perform spermatogenesis implies a significant importance [17].

Congested blood vessels with neutrophils in their lumen that were recorded in the testes (Fig. 3) may be due to relaxed smooth muscle of blood vessels induced by the NO, a free radical. This idea agrees with Pierce *et al*. [28] who recorded that over production of NO can cause toxic effects and vasodilatation effects. Moreover, the pathological lesions in reproductive tract are resulting from lipid peroxidation, inhibition protein synthesis and depletion of ATP by NOS [29].

The current result showed that the serum levels of Testosterone, sperm motility and concentration in immunized and immunized-TMX mice were significantly higher than those in mice treated with TMX only. This result may give an indication that immune response may stimulate enzymatic antioxidant against toxic effects of TMX. This observation is consistent with Marri and Richner [30] who showed that birds can eschew stress caused by a transient increase in ROS generated by immune activation. Coinciding to present results, Colleen *et al*. [31] found that GnRH (FSH, LH), E2 and progesterone with vaccination can increase reproductive steroid hormonal levels, and the total per ejaculate sperm number was correlated with testosterone concentration [32].

The present finding explained that the serum levels of Testosterone, sperm motility and concentration in mice infected with Brucella were lower comparable with other groups. This may indicate a severe degree of infection of the testes. Several infectious conditions resulted in epididymitis and orchitis in male [33]. These results are in consistence with Depuydt *et al*. [34], who showed that ROS might be increased in chronic urogenital diseases associated with an increase in leukocyte counts and the involvement of cytokines associated with epididymitis that affect sperm function. Numerous studies have investigated the relationship between interleukin concentration, WBCs, and sperm function [34]. These epididymal changes are often associated with slight to moderate testicular degeneration and interstitial localization of lymphocytes and macrophages accompanied by neutrophils accumulation in the lumen of the epididymis [35]. These lesions contribute to poor spermatic quality and an increased frequency of degeneration defects associated with infection [36].

5. Conclusion

In conclusion, thiamethoxam possesses a deteriorative toxic effect on spermatogenesis in mice. Therefore, the use of

thiamethoxam should be limited within a designed program.

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