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Impact of the Neonicotinoid Insecticide, Thiamethoxam, on Some Reproductive Parameters in Adult Male Rabbits

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Abstract

Thiamethoxam (TMX**)** is one of new class neonicotinoid insecticides currently available in the Egyptian pesticide markets. The current study was designed to investigate the toxic effect of TMX on reproductive system in adult male rabbits. For this purpose, sixteen adult male *Chinchilla* rabbits were divided into two equal groups. Animals in the first group were treated orally with TMX at dose of 250 mg/kg b.wt for 90 days. The second group was served as control. Hormonal analysis revealed that, TMX induced a significant elevation in the serum testosterone level, while the concentrations of FSH and LH hormones did not exhibit any alterations between treated and control groups. Obtained results also showed that TMX treatment did not cause significant sperm DNA fragmentation, but caused marked alteration in sperm parameters (e.g. concentration, motility and morphology). Degeneration and necrosis of the spermatogenic cells in addition to intertubular edema and vacuolations were the major observed histopathological lesions in the testis of intoxicated animals. In conclusion, administration of TMX for 90 days in male rabbits induced a noticeable adverse effect on the testicular function and structure without the impairment of gonadotropins secretion.

1. INTRODUCTION

Neonicotinoids are a class of neuroactive insecticides chemically related to nicotine **(**Kimura-Kuroda et al., 2012). Currently, they are one of the most widely used insecticides in the Egyptian field, because they have high selectivity towards invertebrate over vertebrate organisms (Lundin et al., 2015). Thiamethoxam (TMX) is one of the second generation neonicotinoids that bind selectively and strongly to nicotinic acetylcholine receptors (nAChRs) of insect than to those of mammals (Goulson, 2013). Depending on data which suggested that some nAChRs subunits are expressed in human testis (α5 and β4) and prostate (α 5) (Flora et al., 2000), and also in rabbit and mouse sperm (α7) (Meizel, 2004; Bray et al., 2005), some researchers investigated the adverse effects of clothianidin, neonicotinoid other than TMX, on the reproductive system in male rats (Bal et al., 2013) and male quails (Tokumoto et al., 2013), while data related to reproductive toxicity of TMX is still scanty and restricted in rats (Rose, 2012). On the other side, it has been established that TMX metabolites have the potential to cause hepatotoxicity in mice. In contrast, there were no increases in hepatic lesions incidences in rats indicating the presence of marked species differences in response to TMX toxicity (Pastoor et al., 2005).

In the light of above described data, there is thus rising concern about unpredictable adverse effects of

TMX and the urgent need for the evaluation of TMX reproductive toxicity in animals models rather than rat and mice. Therefore, a subchronic 90-day oral toxicity study was constructed to investigate adverse effects of TMX on reproductive parameters in adult male rabbit.

2. MATERIALS AND METHODS

2.1. Insecticide: A commercial formulation of thiamethoxam 25% (Actara®, Syngenta Canada Inc.), with chemical name *3-(2-chloro-1,3-thiazol-5-ylmethyl-(5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine*, was purchased from local market of pesticides.

2.2. Animals and treatment protocol: A total of 16 clinically healthy adult male *Chinchilla* rabbits (age: 6.0-6.5 months; wt: 2.5-2.8 kg) were obtained from a commercial rabbit farm at El-Behera Governorate, Egypt. Animals were housed in metallic batteries in a room with 12-hour day/night cycle, a temperature of 24±0.5°C and a relative humidity of 45-65%. Rabbits were fed on a balanced commercial diet (protein is not less than 19%) *ad-libitum*. Animals were kept under supervision without any type of treatment for 2 weeks before the beginning of the experiment for any abnormal signs and for accommodation to laboratory condition. Then, they were randomly divided into 2 groups of each containing 8 rabbits. The first group was served as control and was orally administered distilled water using plastic syringe, while the second group (treated group) was received TMX (diluted with distilled water) at dose of 250 mg/kg b.w., 5 consecutive days per week for 90 days. The selected dose represents about $^{1}/_{20}^{th}$ of oral LD_{50} in rabbits according to Material Safety Data Sheet prepared by Syngenta Canada Inc. The dose was adjusted daily according to body weight changes of individual animal.

2.3. Sampling:

2.3.1. Blood sampling

Blood samples were collected from the ear vein without anticoagulant then centrifuged at 3000 rpm for 10 minutes to separate serum. Serum was stored at –20°C until hormonal analysis.

2.3.2. Semen collection

At the end of the experiment, Semen was collected from bucks via artificial vagina (IMV, l'Aigle Cedex, France). Immediately after collection, semen was kept at 35°C in water bath in order to be evaluated.

2.3.3. Tissue samples

Rabbits were sacrificed by slaughtering and their testes were removed immediately, rinsed in normal saline and fixed in 10% neutral-buffered formalin until use for histopathological examination.

2.4. Hormonal Assay:

The serum level of follicular stimulating hormone (FSH) and luteinizing hormone (LH) was measured by a commercial ELISA kits (CUSABIO® rabbit FSH and LH kits, China) and using ELISA reader ELX808, Biotek, USA. Testosterone hormone was determined using testosterone kit (Immunotech Beckman Coulter Company-USA), according to the standard protocol provided with each kit.

2.5. Sperm parameters:

All sperm analyses were performed by using methods described by El-Battawy and El-Nattat, (2013). Visual motility was evaluated by placing a drop of the diluted semen sample (in saline) on a pre-warmed clean glass slide and examined under heated stage microscope. Sperm motility was recorded at 400x magnification based on the visual estimation of the percentage of sperm possessing progressive motility. Sperm concentration was determined using hemocytometer in a 1:200 dilution. Sperm morphology and mortality was assessed using eosin-nigrosin stain. A film from stained semen drop was spread on a dry clean slide and observed under high power lens. A total of 300 spermatozoa were examined on each slide and then the rates of abnormal and dead spermatozoa were expressed as percentage.

2.6. Analysis of sperm DNA fragmentation by comet assay:

The alkaline (pH >13) comet assay was performed according to the method described by Tice et al. (2000) and El-Ghor et al. (2014). A 1µl aliquot of cell suspension containing approximately 10,000 sperm cells was mixed with 80 µl of 0.5% low melting point agarose (Sigma) and spread on a fully frosted slide pre-dipped in normal melting agarose (1%). After solidification, the slides were placed in cold lysis buffer (2.5-M NaCl,100-mM EDTA, and 10-mM Tris, pH 10) with freshly added 10% DMSO and 1% Triton X-100 for 24 h at 4°C in dark. Then, the slides were incubated in fresh alkaline buffer (300-mM NaOH and 1-mM EDTA, pH >13) for 20 min for unwinding of DNA. The unwinded DNA was electrophoresed for 20 min at 300 mA and 25 V (0.90 V/cm) and neutralized in 0.4-M Trizma base (pH 7.5), then, finally fixed in 100% cold ethanol, air dried, and stored at room temperature

until they were scored. The degree of DNA migration for each sample was determined by simultaneous image capture and scoring of 100 cells at ×400 magnification using Komet 5 image analysis software developed by Kinetic Imaging, Ltd (Liverpool, UK). The extent of DNA damage was evaluated according to the following endpoints measurements: *Tail length*: used to evaluate the extent of DNA damage away from the nucleus and expressed in µm; % *DNA in tail*: Intensity of all tail pixels divided by the total intensity of all pixels in the Comet; and *tail moment*: calculated as: tail moment = tail length × % DNA in tail/100.

2.7. Histopathological examination

Portions of the preserved testis were processed and embedded in paraffin wax. Sections of 5-6 Microns were made and stained routinely with hematoxylin and eosin (H&E) according to Bancroft and Gamble, 2008.

2.8. Statistical analysis

Data are represented as mean ± standard error of mean (SEM). The SPSS/Program (version 21.0; SPSS, Inc.) was used for the statistical analysis. Samples-independent t-test was used to determine differences between groups in all parameters. Values of $P<0.05$ were considered to be statistically significant.

3. RESULTS

3.1. Clinical observation

There is no major signs of toxicity were observed except diarrhea which appeared periodically in some of TMX-treated animals shortly after dosing and cured spontaneously. Another interesting observation is the increase of libido among treated bucks in comparing with control. Increase in libido was manifested by the rapidity of semen collection and shorter teasing time.

3.2. Hormonal assay

There were no significant differences between control and treated group in serum FSH and LH levels. While, testosterone level showed a significant elevation in intoxicated animals in comparing with control group as shown in table 1.

3.3. Ejaculated sperm analysis

As shown in table 2, there were significant modifications ($p<0.05$) in sperm parameters in the TMX-treated group. These modifications include a decrease in sperm motility (both mass and individual) and count, increase in the percentage of dead spermatozoa and sperm deformities comparing with control group.

3.4. Sperm DNA fragmentation assay (Comet assay)

Results represented in table (3) and figure (1) showed that, although TMX caused a numerical increase in tail length, DNA (%) in tail and tail moment, these differences did not reach statistical significance.

3.5. Histopathological findings

Histopathological examination of testis in control animals showed normal seminiferous tubules with active spermatogenesis (Fig. 2). While, testis of TMX-intoxicated rabbits showed distortion, degeneration and necrosis of the spermatogenic cells in seminiferous tubules and intertubular edema (Fig. 3), in addition to excess intratubular vacuolations and severely dilated intertubular arteriole (Figs. 4,5).

Table 1. Effect of TMX on serum levels of testosterone, FSH and LH.

	Testosterone (ng/ml)	FSH (mlU/ml)	LH (mlU/ml)
Control	1.40±0.19	2.31±1.20	1.73±0.30
Treated	3.16±0.61*	2.21±0.56	1.06±0.48

Data are expressed as mean ±SEM. ($n=8$)
Mean differences between values bearing asterisk within the same column are statistically significant ($p<0.05$).

Table 2. Effect of orally administered TMX on sperm parameters.

	Individual motility (%)	Mass motility (%)	Dead (%)	Deformity (%)	Sperm count (Million/ml)
Control	85±00	81±2.4	4.8±.49	18.4±.98	737.7±64.4
Treated	25.5±7.28*	28±5.7*	28.7±2.8*	39.2±4.8*	292.4±67.8*

Data are expressed as mean±SEM. (*n=8*)
Mean differences between values bearing asterisk within the same column are statistically significant ($p<0.05$).

Table 3.Effect of TMX on the sperm DNA fragmentation parameters (Comet assay).

	Head diameter (px)	%DNA in sperm head	Tail length (px)	%DNA in sperm tail	Tail Moment
Control	27.06±1.3	73.13±2.23	4.64±0.64	26.84±2.23	1.63±0.30
Treated	28.80±1.0	72.30±2.08	5.66±0.83	27.70±2.08	1.85±0.36

Data are expressed as mean±SEM

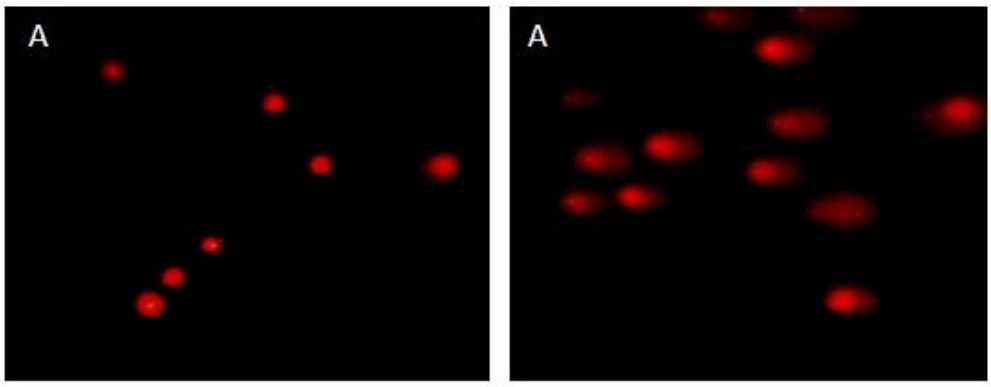

Fig.1: Effect of TMX on sperm DNA fragmentation. **(A)**: Control; **(B)**: Treated

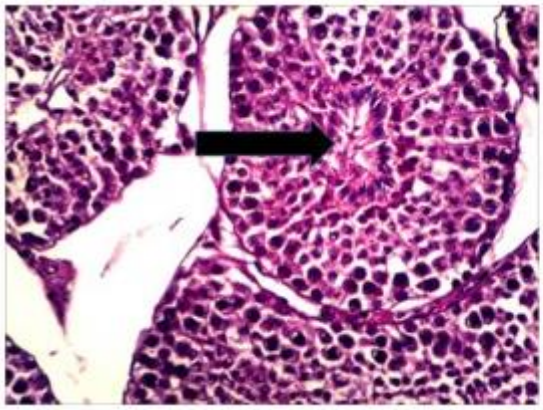

Fig. 2: Testis of control rabbit: Higher magnification to show numerous impacted tubules with the normal active spermatogenic and spermatozoal contents (arrow). H&E, X 400

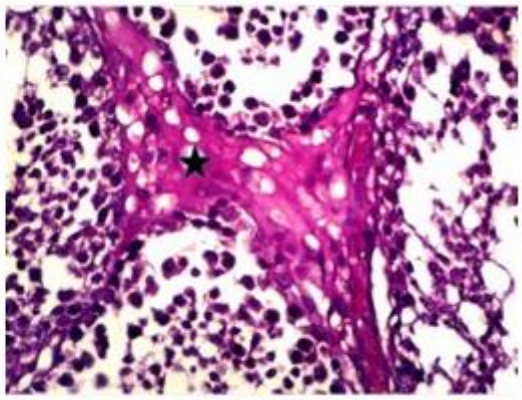

Fig. 3: Testis of TMX-intoxicated rabbit: Most of the seminiferous tubules affected by distortion, degeneration and necrosis of the spermatogenic cells in addition to intertubular edema (asterisk). H&E, X 400

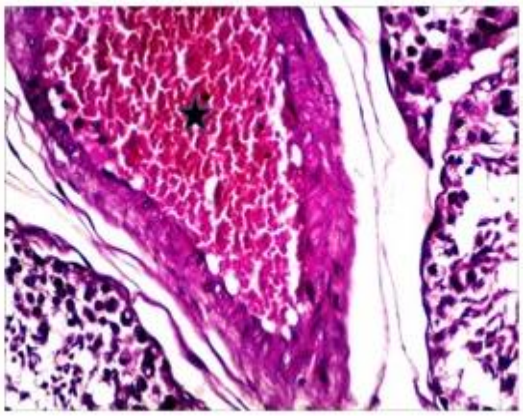

Fig. 4: Testis of TMX-intoxicated rabbit: Higher magnification to show one severely dilated and congested intertubular arteriole (asterisk). H&E, X 400.

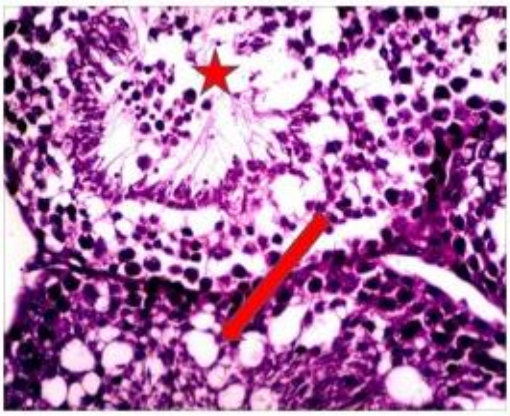

Fig. 4: Testis of TMX-intoxicated rabbit: Higher magnification to show one severely dilated and congested intertubular arteriole (asterisk). H&E, X 400.

4. DISCUSSION

Thiamethoxam (TMX) is a novel neonicotinoid insecticide has the potential to induce impairment of many physiological parameters and histological structures in albino rats (Shalaby et al., 2010). To our knowledge, this is the first report about the adverse effects of this insecticide on the reproductive system of adult male rabbit. To ensure that all possible adverse effects are expressed and to detect the maximum effect of any drug or chemical on sperm quality in rabbit, at least 61 days of exposure is necessary (period of six cycles of seminiferous epithelium) (Klinefelter and Rao Veeramachaneni, 2014). So, the duration of current study was therefore set at 90 days.

Considering hormonal alterations, it was clear that oral administration of TMX for 3 months induced a significant elevation in testosterone level without modification in FSH and LH concentration. This increase in testosterone was reflected behaviorally in treated rabbits by the increase of libido which observed during semen collection. Also, this elevation is not related to the insecticide effect on pituitary gland and originates directly from testis. This understanding may attributable to hyperplasia of leydig cells and/or over secretion as evidenced histopathologically. Since neonicotenoids are nicotine agonist (Tomizawa and Casida, 2005) and has effects like nicotine (Kimura-Kuroda et al., 2012), nicotine was previously demonstrated to have no association between its

exposure and induction of Leydig cell hyperplasia in humans, but it was able to induce Leydig cell tumor in rats (Cook et al., 1999). In the same context, sub-chronic exposure to neonicotinoide insecticide, clothianidin (CTD), did not significantly change serum testosterone level or sperm parameters, but caused significant decreases in weights of epididymis, right cauda epididymis and seminal vesicles in adult male rats (Bal et al., 2013). This variation in finding could be explained in the light of sensitivity differences between rats and rabbits.

Among all measured parameters in our study, the more pronounced alterations were seen in ejaculated sperm characteristics. TMX exposure significantly decreases sperm count and motility, while percentage of sperm mortalities and deformities were significantly increased. Similar results were demonstrated in developing male rats were exposed to CTD (Bal et al., 2012). Also, the exposure of adult male rats to nicotine resulted in alterations in sperm characteristics (Oyeyipo et al., 2011). In *vitro* testing, the fertilization ability of mouse sperm was adversely affected by the direct exposure to acetamiprid and imidacloprid (two major members in the family of neonicotinoid pesticides) (Gu et al., 2013) . It is known that the cholinergic system is present within the mammalian testis and functional AChRs are found on male germ cells and Sertoli cells (Schirmer et al., 2011). Therefore, Bray et al. (2005) suggested that sperm nAChRs play an important role in the control of its motility. Mice lacking in nAChRs subtypes or with reduced acetylcholine (ACh) levels showed reduced sperm motility. We can hypothesize that the disturbed function of rabbit testicular tissue may be related directly to its effect on reproductive nicotinic system. The detected impairment of testicular function in the current study was associated with structural lesions as that some affected tubules were with less spermatogenesis, presence of either excess intratubular vacuolations or some exfoliated necrotic immature spermatogenic cells, in addition to distortion, degeneration and necrosis of the spermatogenic cells, intertubular edema and congested intertubular arteriole. It was established that testis of male mice were exposed to CTD and environmental stress showed vacuolated seminiferous epithelia (Hirano et al., 2015). Moreover, CTD has the ability to induce vacuolization and DNA fragmentation in seminiferous tubules in male quails (Tokumoto et al., 2013). In rabbits, imidacloprid induced many histological lesions in testicular tissue. The more pronounced alterations were widened interstatial space and hypertrophied leydig cells (Memon et al., 2014).

In addition to the direct effect of TMX on nAChRs, there is another way by which neonicotinoides can affect male fertility. Although the generation of reactive oxygen species (ROS) by mammalian spermatozoa is very important in the control of normal sperm function (Aitken, 1995), there is now good evidence to indicate that free radicals are involved in DNA damage and apoptosis of spermatozoa, seen in CTD-treated male rats (Bal et al., 2013). This may be attributable to the high content of poly unsaturated fatty acids (PUFAs) in mammalian spermatozoa. Therefore, ROS can attack the unsaturated bonds of lipids in the sperm membrane and destroys the structure of lipid matrix in the membranes of spermatozoa resulting in axonemal damage, decreased sperm viability and increased mid-piece morphological defects and completely inhibits spermatogenesis in extreme cases (Aitken and Roman, 2008; Kothari et al., 2010).

Comet assay showed that sperm DNA fragmentation parameters were increased in male rabbits after 90-day of TMX treatment, but this change was did not statistically significant. This finding is compatible with those obtained by Bal et al. (2013) which demonstrated that CTD did not cause sperm DNA fragmentation in adult male rat. These data indicated that TMX is unlikely to be genotoxic.

5. CONCLUSION

It could be concluded that the prolonged exposure to TMX can markedly alter function of testis and to lesser extent testicular histology without impairment of gonadotropins secretion and sperm genetic material in adult male rabbits. Thus, TMX may pose reproductive risks on mammalian reproductive health.

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