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Thiamethoxam Induced Oxidative Stress and Histopathological Alterations in Male Patients

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ABSTRACT: Thiamethoxam is one of the second-generation neonicotinoids, a new class of insecticides. The study was designed to investigate the effect of thiamethoxam on fertility in bucks. For this purpose, sixteen male adult male chinchilla rabbits were divided in two groups. Thiamethoxam intoxicated group was treated with thiamethoxam at dose of 250 mg/Kg body weight for 3 months. Semen analysis revealed distinct changes in sperm characteristics including significant decrease in sperm motility (both mass and individual) and sperm count. In addition, significant increase in dead spermatozoa and sperm deformities. Moreover, elevation in testicular concentration of MDA and GST was significant. GSH was significantly decreased. Degeneration and necrosis of spermatogenic cells with intertubular edema and vacuolations in seminiferous tubules were the major observed histopathological changes in the testis of intoxicated animals. In conclusion, thiamethoxam administration for 3 months induced significant decrease in fertility and oxidative stress in the reproductive system of adult male.

KEY WORDS: Antioxidants, Oxidative, Patients, Stress.

INTRODUCTION

Oxidative stress is overproduction of free radicals including reactive oxygen species and reactive nitrogen species or inadequate antioxidant defense mechanism in tissues. Free radicals in reproductive organs lead to severe cytotoxic effects including lipid peroxidation, DNA damage, Degeneration, apoptosis, antioxidant depletion, impaired spermatogenic process and infertility [1, 2].
Neonicotinoids are novel class of insecticides, widely distributed over the world and registered in over 120 different countries. They were discovered in 1990 and have grown rapidly with annual sales more than $3.5 billion. NEOs are considered neurotoxicants because they have agonist action on nicotinic acetylcholine receptors both in mammals and insects. They dominated the insecticide market in a short time because they selectively bind to insect nAChRs with reduced action on vertebrate nicotinic receptors. Neos include seven compounds arranged in three generations: first generation (Imidacloprid, Nitenpyram, acetamiprid, thiacloprid), second generation (thiamethoxam and clothianidin) and third generation Neos (dinotefuran) [3, 4].
Although this common belief about their lower toxicity in mammals, there is great evidence that NEOs has toxic effect on non-target species. Clothianidin was reported to decrease weight of reproductive organs significantly and increase testicular lipid peroxidation in a dose dependent manner. El okle et al. [5] postulated that thiamethoxam at a dose of 100 mg/Kg body weight for 7 days induced sloughing and depletion of germinal cells in seminiferous tubules in rat. In another study, oxidative stress was increased in the liver with potential risk of hepatocarcinogenicity in rat [6]. However, it was reported that hepatocarcingenicity occurred in liver of mice rather than in rat. this was explained by species specificity in the metabolism of TMX [7, 8].
Although neonicotinoids share the same characters and properties [9], previous studies of neonicotinoids did not provide detailed information about thiamethoxam toxicity in rabbits. Therefore, the scope of the present study is to explore short term toxic effect of TMX on sperm characteristics, oxidative stress, spermatozoa DNA fragmentation, as well as on histopathological structure of testis.

MATERIALS AND METHODS

Chemicals and reagents

A commercial thiamethoxam 25% WS (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 a local insecticide market in Egypt. Biochemical diagnostic kits for GST, GT, MDA, and catalase enzyme were obtained from Biodiagnostic co. (Egypt). Other chemicals and reagents used for sperm analysis, comet assay and histopathological analysis were of the best available pharmaceutical grade.

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Preparation of Thiamethoxam

For preparation of stock solution of thiamethoxam, 10 grams of Actara® 25% were dissolved in 30 ml of distilled water. Each rabbit in the treated group received 3 ml of the stock solution per kg which is equivalent to 250 mg/kg.

Animals and experimental design

Twenty 20 adult male Chinchilla rabbits (6.0-6.5 month's old) weighing 2.5-2.8 kg were obtained from a commercial rabbit farm in Egypt. Animals were housed in a metallic battery in a room with 12-hour day/night cycle, a temperature of 24 ºC, and humidity of 45–65%. During the experiment, animals were provided with a balanced commercial ration ad-libitum with protein not less than 19%. Also, fresh distilled water was given ad-libitum. Animals were kept for 14 days without any treatment prior dosing for acclimatization to laboratory conditions. Animals were randomly divided into 2 groups with eight animals in each group. First group was taken as control group, received distilled water 5 days in a week. the second group served as treatment group, received thiamethoxam at dose 250 mg/kg body weight for 5 days in a week for 3 months. The dose of thiamethoxam was adjusted on daily basis according to body weight of every animal. The selected dose of thiamethoxam represents about 1/20 LD50 of thiamethoxam in rabbit, revealed in the material safety data sheet prepared by Syngenta [10]. To see the maximum effect of any drug on sperm quality in rabbit at least 65 days are necessary because the spermatogenic cycle is about 10.7 days in rabbits and the treatment should be continued for a minimum of six cycles of the seminiferous epithelium prior to termination of the study to ensure that all possible adverse effects are expressed [11].

At the end of experiment spermatozoa was collected from rabbits of each group through artificial vagina (IMV, l'Aigle Cedex, France). Immediately after collection, semen was divided into two parts. The first part kept at 37 °C in water bath to evaluate parameters of spermatozoa while the other part kept frozen at -20 ºC till used for the assessment of genotoxicity using comet assay. After semen collection, animals from both groups were euthanized. Right testicles were rinsed in normal saline solution then, fixed in 10 % neutral buffered formalin for histopathological examination. The other testicles were stored at -70 ºC until use for oxidative stress analyses (GST, GSH, MDA and catalase).

Sperm analyses

All sperm parameters were performed according the method described by [12]. Freshly collected spermatozoa was used for analyses. Mass and individual motility of spermatozoa were determined by light microscope with a heated stage. To determine sperm viability and percentage of morphologically abnormal spermatozoa, stained slides with eosin-nigrosin (1.67% eosin, 10% nigrosine and 0.1 M of sodium citrate) were prepared. Then slides were examined at 400x under light microscope. The sperm was considered dead if the stain was absorbed and sperm was stained pink. A total of 300 spermatozoa were examined in different fields on the slide and percentage of dead and abnormal spermatozoa were calculated and expressed as a percentage.

Estimation of testicular oxidative/antioxidative biomarkers

Oxidative stress in testicular tissue was evaluated by measuring testicular concentration of GSH and thiobarbituric acid reactive substances (TBARS), also the testicular antioxidant enzymes glutathione-S-transferase (GST) and catalase (CAT). GSH level in testicular tissue homogenate was measured spectrophotometrically according to the method of [13]. This method is based on the reduction of 5,5'dithiobis (2-nitrobenzoic acid) (DTNB) by GSH to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration, and its absorbance was measured at 405 nm. GST activity in tissue homogenate was measured spectrophotometrically according to the method demonstrated by [14], which is based on measuring the conjugation of 1-chloro-2,4- dinitrobenzene (CDNB) with reduced glutathione. The rate of conjugation is directly proportional to the GST activity in the sample. Catalase concentration was measured on the basis that catalase reacts with a known amount of H_2O_2. Then the reaction has been stopped after 1 minute by a catalase inhibitor. In the presence of peroxidase (HRP), remaining H_2O_2 reacts with 3, 5-Dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with color intensity inversely proportional to the amount of catalase in the original sample [15, 16]. MDA concentration was detected according to the method described by [17, 18]. The assay depends on that thiobarbituric acid (TBA) reacts with MDA in acidic medium at temperature of 95 ºC for 30 minutes to form thiobarbituric acid reactive product chromophore with absorbance at 532 nm. Values of thiobarbituric acid referred as MDA because MDA is produced upon decomposition of polyunsaturated fatty acid (PUFAs).

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Genotoxicity investigation of spermatozoa using comet assay

The assay was carried out the method performed by [19] and [20]. In brief, the samples were prepared by centrifugation at 700 x g for 2 minutes then supernatant was discarded, and sediment washed and suspended in Hank's balanced salt solution (HBSS) (sodium chloride 8000mg, potassium chloride 400 mg, potassium phosphate monobasic 60 mg, glucose 1000 mg, phenol red Na salt 10 mg, sodium phosphate dibasic 48 g, magnesium phosphate anhydrous 98mg, calcium chloride anhydrous 140 mg, sodium bicarbonate 350 mg). 10 µl of cell suspension was mixed with 0.5 % low melting point agarose then spread on a frosted slide. After solidification, the slides were placed in a cold lysis buffer then 10% DMSO and 1% triton were added then the slides were incubated for 24 h at 4 c in dark. For unwinding of DNA, slides were incubated in fresh alkaline buffer for 20 minutes. Then, DNA was electrophoresed for 20 min at 300 mA and 25 V. Scoring of DNA migration was carried out using Komet 5 image analysis software developed by Kinetic Imaging, Ltd (Liverpool, UK).

DNA damage was evaluated by the following endpoints measurements: tail length which 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; tail moment: calculated through by this equation tail length × %DNA in tail/100.

Histopathological study

The fixed testicular tissues were dehydrated in ethyl alcohol with ascending concentration. Then specimens were cleared with xylene and embedded in paraffin. Finally, embedded tissues were sectioned by microtome into 4 µm thickened sections and stained with hematoxylin and eosin stains for histopathological evaluation [21].

Statistical analysis

Data were analyzed using SPSS/Program (version 21.0; SPSS, Inc.). samples independent t-test was used to determine difference between groups. Results were considered statistically significant if P value was <0.05. Data were expressed as mean±SEM (standard error of mean).

RESULTS

Effect of TMX on characteristics of spermatozoa

Sperm characteristics of control and TMX-intoxicated group are shown in table 1. there were significant modifications *(p<0.05)* of sperm parameters in rabbits after 90 days of TMX administration. These modifications include significant decrease in sperm motility (both mass and individual) and sperm count. Regarding percentage of dead spermatozoa and sperm deformities, there were significant increase in TMX-intoxicated group comparing with control group.

Table 1: Effect of TMX on sperm parameters.

Group	Individual motility (%)	Mass motility (%)	Dead (%)	Deformity (%)	Concentration (Million/ml)
Control	85.0± 00	81.0± 2.4	4.8± 0.49	18.4± 0.98	737.7± 64.4
Treated	25.5± 7.28*	28.0± 5.7*	28.7± 2.8*	39.2± 4.8*	292.4± 67.8*

Data were expressed as mean ± SEM.

Mean differences between values bearing asterisk within the same row are statically significant *(p<0.05).*

Effect of thiamethoxam on testicular oxidant/antioxidant indices

Regarding testicular oxidative stress table 2 shows significantly increased concentration of MDA *(p<0.05)* (110.29 ± 17.24 nmol/g tissue) in testicular tissue and significant increase in the activity GST *(p<0.05)* (11.18 ± 0.69 U/g tissue) in TMX-treated group in comparison with control group. However, GSH concentration in the treated rabbits with TMX was significantly decreased (0.05 ± 0.01 mmol/g tissue, *p<0.05)* compared to the control group (0.15 ± 0.03 mmol/g tissue).

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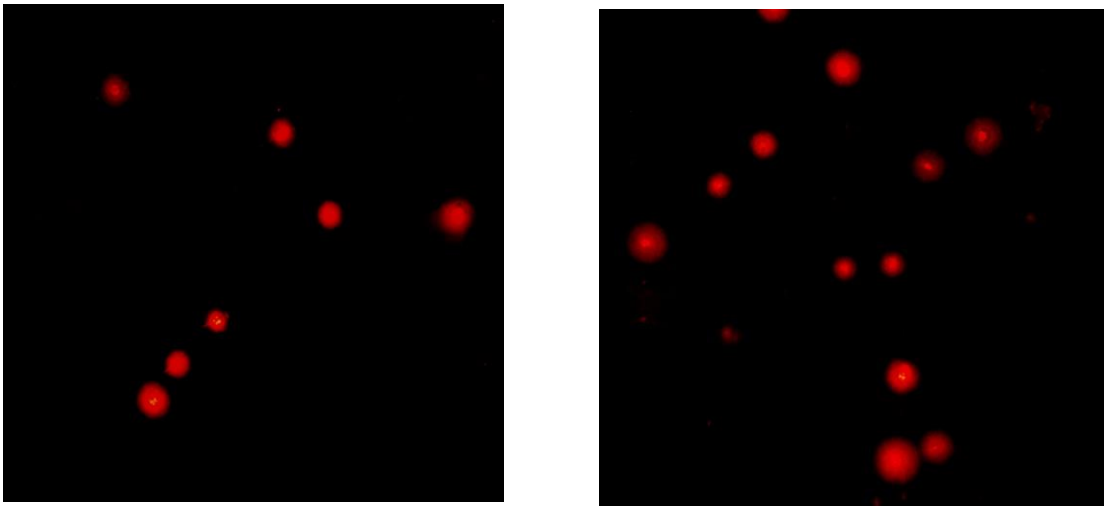
Table 2: Effect of thiamethoxam on oxidative stress biomarkers in testicular tissue of adult male rabbit.

Group	GSH (mmol/g tissue)	Catalase (U/g tissue)	GST (U/g tissue)	MDA (nmol/g tissue)
Control	0.15 ± 0.03	0.60 ± 0.16	9.14 ± 0.43	49.24 ± 15.48
Treated	0.05 ± 0.01*	0.51 ± 0.21	11.18 ± 0.69*	110.29 ± 17.24*

Results were expressed as mean ±SEM. (n=8).
Mean differences between values bearing asterisk within the same row are statically significant *(p<0.05)*.

Sperm DNA fragmentation by comet assay

Results are presented in figure 1 and table 3. Although TMX induced numerical increase in parameters of sperm DNA fragmentation in TMX-intoxicated group, these differences did not reach statistical significance in comparison with control group.



Spermatozoa of control rabbit **Spermatozoa of TMX treated rabbit**

Figure 1: Effect of TMX exposure on DNA fragmentation of spermatozoa of adult male rabbits.

Table 3: Effects of TMX on the spermatozoa DNA Damage (Comet Assay) Induced in adult male rabbit semen.

Parameters	Control	Treated
Head Diameter (px)	27.06±1.327	28.80±1.02
%DNA in Head	73.13±2.23	72.30±2.08
Tail Length (px)	4.64±.644	5.66± .83
%DNA in Tail	26.84±2.23	27.70±2.08
Tail Moment	1.63±.301	1.85± .36

Results were expressed as mean ± SEM. (n=8).

Histopathological studies

In comparison with histologically normal testicular sections of control group, testicular sections of TMX-treated group showed distortion, degeneration, and necrosis besides intertubular edema (fig. 3). Moreover, severely dilated, and congested arteriole was notified in TMX-treated group (Fig. 5). Furthermore, spermatogenic process was affected negatively and intratubular vacuolations were also observed (Fig. 4)

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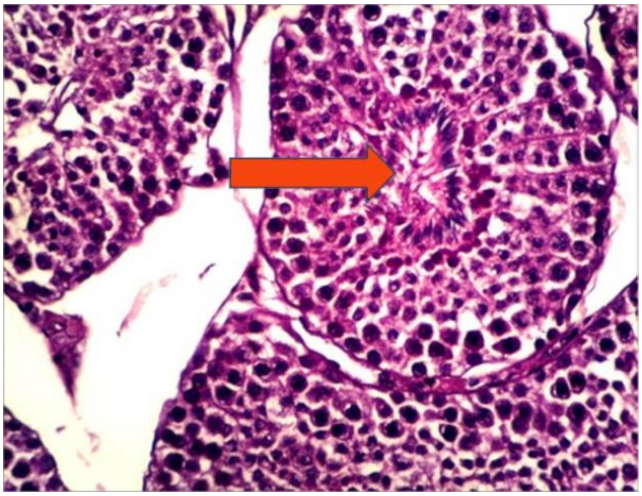

Figure 2: Testis of control rabbit: Higher magnification to show numerous impacted tubules with the normal active spermatogenic and spermatozoal contents (arrow). H&E, X 400

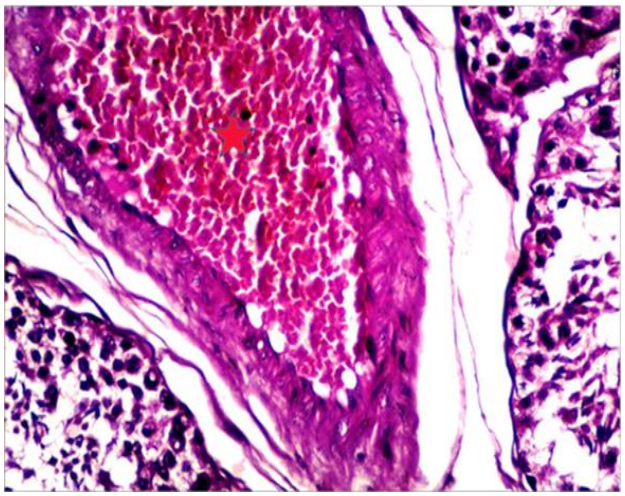

Figure 3: Testis of intoxicated rabbit with TMX: Most of the seminiferous tubules affected by distortion, degeneration, and necrosis of the spermatogenic cells in addition to intertubular edema (asterisk). H&E, X 400.

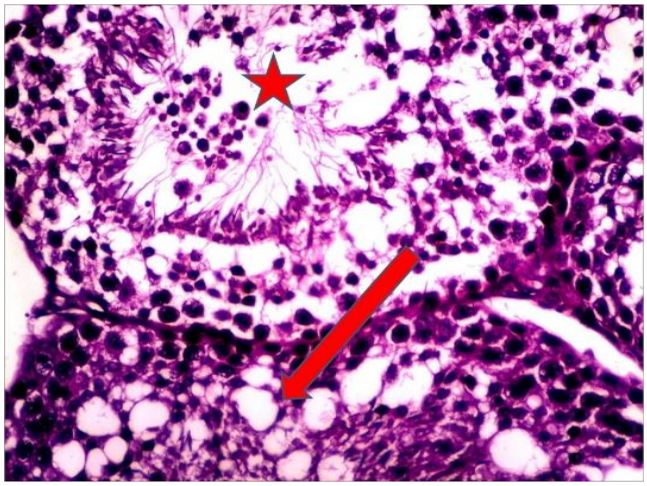

Figure 4: Testis of intoxicated rabbit with TMX: Some affected tubules with less spermatogenesis and presence of either excess intratubular vacuolations (arrow) or some exfoliated necrotic immature spermatogenic cells (asterisk). H&E, X 400.

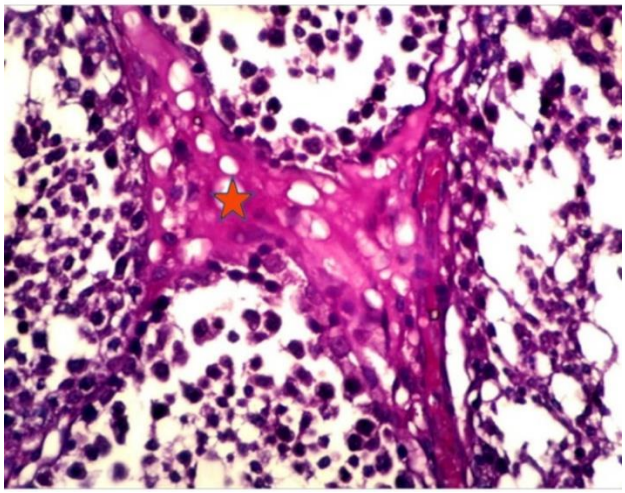

Figure 5: Testis of intoxicated rabbit with TMX: Higher magnification to show one severely dilated and congested intertubular arteriole (asterisk). H&E, X

DISCUSSION

Thiamethoxam (TMX) is a new neonicotinoid insecticide evidenced to induce impairment of many physiological parameters and histological structures in albino rats [22]. Also in human, there reports about NEOs intoxication with clinical manifestations including nausea, vomiting, drowsiness, disorientation, dizziness, oral and gastroesophageal erosions, hemorrhagic gastritis, productive cough, fever, leukocytosis, muscle weakness, hypothermia, and convulsions [23-25]. NEOs were classified by EPA as toxicity class II and/or class III agents [26]. The results presented in the current study demonstrate that ingestion of TMX for 90 days induced some adverse effects, to limited extents, in the male reproductive system in rabbits.

The more pronounced alterations were seen in ejaculated sperm characteristics. TMX exposure significantly decreases sperm count and motility both mass and individual motility, while percentage of dead spermatozoa and deformities were significantly increased. These results were similar to those reported by [27] and [28] following exposure of developing male rats to CLO and imidacloprid.

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In the line with the above, the exposure of adult male rats to nicotine resulted in alterations in sperm characteristics [29]. Furthermore, In vitro 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) [30]. 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 [31]. Therefore, [32] proposed that sperm nAChRs play an important role in the control of its motility. This was emphasized by using nAChRs knockout mice. The motility of spermatozoa was severely affected in this type of mice. We can hypothesize that the disturbed function of rabbit testicular tissue may be related directly to its effect on reproductive nicotinic system. This assumption needed further studies.

The detected impairment of testicular function in the current study was associated with structural lesions as 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. [33] postulated that testis of male mice which were exposed to CTD, and environmental stress showed vacuolated seminiferous epithelia. Moreover, CTD induced vacuolization and DNA fragmentation in seminiferous tubules in male quails [34]. In another study in rabbits, imidacloprid induced many histological lesions in testicular tissue. widened interstitial space and hypertrophied Leydig cells were primarily observed in this study [35]. The proposed histopathological impairment of the testicular tissue may be attributable to generation of other toxic substances as formaldehyde (HCHO) and further N-methylol intermediates as result of cytochrome mediated oxidative cleavage of oxadiazine ring of TMX so TMX is considered as "formaldehyde generator" [36, 37]. The hepatic carcinogenicity of TMX in mice was confirmed by generation of formaldehyde during its metabolism [38]. As demonstrated, this can be regarded as an alternative mechanism of testicular toxicity.

In addition to the hypothesized effect of TMX on nAChRs, there is another hypothesis by which neonicotinoids 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 [39], there is now good evidence that free radicals can cause DNA damage and apoptosis of spermatozoa. This was seen in CTD-treated male rats [40]. ROS are small, oxygen-based molecules that are highly reactive because of unpaired electrons such as superoxide anion, hydrogen peroxide, hydroxyl ion and peroxynitrite. Oxidative stress results from imbalance between the production of ROS and their removal by the available antioxidant system. The major antioxidant enzymes are catalase, GPX, GST and sorbitol dehydrogenase (SOD) [41]. It is reported that NEOs induce their toxic effects by the oxidative stress. The oxidative stress occurred because NEOs have the potential to inhibit Δ-aminolevulinate dehydratase (δ-ALA-D), a metalloenzyme with thiol group (SH group) and require zinc (Zn) for its activity so this enzyme is easily oxidized by many chemicals. The inhibition of this enzyme results in accumulation of his substrate, aminolevulinic acid (ALA). ALA is a pro-oxidant and weak γ-aminobutyric acid (GABA) agonist, resulting in oxidative stress [42]. There is an alternative way the NEOs induce oxidative stress. Nitic oxide is a physiological messenger synthesized by nitric oxide synthase. Activation of nitric oxide synthase by NEOs results in increase of nitric oxide. Consequently, excessive stimulation of nitric oxide synthase leads to massive production and accumulation of nitric oxide and peroxyl nitrite, which contributes greatly to oxidative stress [43].

CONCLUSION

Our research suggested that exposure to TMX for prolonged period has adverse effects on the reproductive function. TMX exert his effect on reproductive system of rabbits by inducing oxidative stress. Consequently, exerting negative effect on sperm parameters without causing DNA fragmentation of spermatozoa. In addition, TMX induced histopathological alterations in testis. Therefore, TMX may poses reproductive risk potential on mammalian reproduction.

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