



SUBCHRONIC ORAL EXPOSURE OF MALE RATS TO THIAMETHOXAM, QUERCETIN AND THEIR COMBINATION ON OXIDATIVE STRESS AND ANTIOXIDANT PARAMETERS IN TESTES (a)

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ABSTRACT

The objective of this study is to determine the subchronic toxicity of thiamethoxam and ameliorative effect of quercetin on oxidative stress and antioxidant parameters in testes of male rats. Subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on oxidative stress and antioxidant parameters in testes was studied. In this study, 96 adult male rats were divided in 6 groups each comprises of 8 rats for 60 and 90 days treatment schedule. Rats in group I were kept as control and received 2% gum Acacia. Rats in groups II, III, IV, V, VI were administered with lower dose of TMX (105 mg/kg b.wt.), higher dose of TMX (210 mg/kg b.wt.), quercetin (50 mg/kg), lower dose of TMX (105 mg/kg) + Qu (50 mg/kg) and higher dose of TMX (210 mg/kg b.wt.) + Qu (50mg/kg), respectively. Rats from each group were sacrificed on 61st and 91st days of experiment. In view of this, effect of subchronic oral toxicity of thiamethoxam and amelioration potential of quercetin was studied on various parameters *viz.* oxidative and nitrosative stress indices and antioxidant parameters in testes. The histopathological alterations in tissues of testes of animals in various treatment groups of 60 and 90 days treatment were studied. The levels of protein carbonyl and nitric oxide in testes were increased significantly ($p < 0.05$) in thiamethoxam treated groups as compared to control and quercetin groups, in 60 and 90 days treatment schedules, indicating oxidative stress, whereas tissue protein showed opposite trend due to protein catabolism induced by thiamethoxam. There was significant increase in the level of tissue protein and decrease in oxidative stress biomarkers described above when TMX was administered with quercetin indicating ameliorative effect of quercetin. The levels of GPx and GSH testes were significantly ($p < 0.05$) lower in TMX treated groups as compared to control, but there was significant restoration of these parameters to normal when TMX was administered along with quercetin indicating amelioration by quercetin. The levels of protein carbonyl content and nitrite in testes increased significantly ($p < 0.05$) whereas tissue protein showed opposite trend due to protein catabolism induced by thiamethoxam indicating oxidative stress. Increased concentration of GPx and GSH level was also observed in this study indicating amelioration by quercetin. Quercetin co-treatment with thiamethoxam groups did not produce improvement in histopathological changes manifested in the testes.

KEYWORDS: Subchronic toxicity, male rats, thiamethoxam, quercetin, oxidative stress

INTRODUCTION

The contribution of agro-chemicals towards increasing agricultural production is well established. All the pesticides, in addition to their target effects, have been found to affect nontarget organisms, including humans (Forti *et al.*, 1993; Chaudhuri *et al.*, 1999). Thiamethoxam is first commercially available second generation neonicotinoid and belongs to thioncotynyl subclass. Neonicotinoids (clothianidin, acetamiprid, thiacloprid, thiamethoxam, dinotefuran and nitenpyram) are a group of systemic insecticides routinely used in modern farming systems to help protect crops such as oilseed rape, maize, sugar beet, sunflowers and potatoes from sap sucking insects such as aphids and other insect (Banerjee *et al.*, 1999; Panemangalore *et al.*, 1999; Hernández *et al.*, 2005). Quercetin acting as free radical scavengers (antioxidant) was shown to exert a protective effect in reperfusion ischemic tissue damage (Fraga *et al.*, 1987; Halliwell, 1994 and Santos *et al.*, 1998). In recent years, natural

antioxidants especially those in natural foods or medicinal plants have drawn attention of many food or medicine researchers. Many insecticides are hydrophobic molecules, which bind extensively to biological membranes, especially to the phospholipids bilayers (Lee *et al.*, 1991). Numerous studies showed that antioxidant substances protect cells against deleterious effects of several environmental agents (Almeida *et al.*, 1998).

Study has shown that quercetin exerted protective effect against irradiation-induced inflammation in mice through increasing cytokine secretion (Jung *et al.*, 2012). Quercetin possesses activity against isoproterenol-induced myocardial oxidative injury and immunity function impairment, and that the mechanism of pharmacological action was related at least in part to the antioxidant activity of quercetin (Liu *et al.*, 2012). Quercetin decreased histological signs of acute inflammation in the treated animals in a dose-dependent manner *via* suppressing leucocyte recruitment, decreasing chemokine levels and

levels of the lipid peroxidation end product of malondialdehyde, and increasing antioxidant enzyme activity in experimental rat model (Dong *et al.*, 2014).

MATERIALS AND METHODS

Ethical approval

The Experiment was approved by the Institutional Animal Ethics Committee constituted as per the article in this study *vide* memo number VPHE/IAEC/1316-36 dated 30/3/2015.

Animals

Wistar rats weighing 120-140 g were housed in polyacrylic cages in group of eight rats per cage in the departmental animal house. Bedding material (rice husk) was changed on alternate days. The animals were provided with feed and water *ad libitum* and maintained at room temperature with a natural light-dark cycle. The Wistar rats were acclimatized to laboratory conditions for 2-3 days before the experiment was conducted. Animal house temperature varied between 22 to 27°C throughout the study.

Drugs and Chemicals

The formulation product of thiamethoxam (ACTARA®) 25G or (25%) (Syngenta India Ltd) and quercetin dihydrate was used in this experiment, gum acacia was used as a vehicle for oral administration of the compounds. Quercetin dihydrate were procured from Sigma-Aldrich Company. 2M HCl solution, 10mM DNPH solution in 2 M HCl solution, 20% Trichloroacetic acid (cold) solution, 1:1 (v/v) mixture of ethanol and ethyl acetate, 6M Guanidine hydrochloride solution in distilled water, 3N HCl solution, 1.0% Sulfanilamide in 3 N HCl solution, 0.1% N-naphthyle thylenediamine dihydrochloride (NEDD) in distilled water, Bovine serum albumin fraction V (BSA) stock solution, 2% sodium carbonate solution, 0.1N sodium hydroxide (NaOH) solution, 1.56% copper sulphate solution, 2.37% sodium potassium tartarate solution, Folin - Ciocalteu reagent solution (1N), 2.0 mM reduced glutathione solution, 0.4 M sodium phosphate buffer solution containing 4×10^{-4} M EDTA, 0.01 M sodium azide (NaN₃) solution, 1.2 mM H₂O₂ solution, 0.4 M disodium hydrogen phosphate (Na₂HPO₄) solution, m-phosphoric acid precipitation solution, DTNB reagent, Phosphate buffer, Reduced glutathione, 1- Chloro-2-4-dinitrobenzene (CDNB), m-phosphoric acid precipitation solution, dithiobisnitro benzoic acid (DTNB) were procured from Sisco and Himedia Research Laboratories Pvt. Ltd. Mumbai, India.

Instruments

Various instruments used in this study like refrigerator (Vestfrost™) deep freezer (-20°C and -80°C, Vestfrost™) centrifuge machine (REMI CPR-30 PLUS) vortex mixture (REMI CM-101 PLUS and SPINIX), weighing balance (SHIMADZU™), spectrophotometer (SYSTRONIC™ INDIA) and tissue homogenizer in the laboratory Department of Veterinary Pharmacology and Toxicology, LUVAS, Hisar were used.

Glasswares and Plastic wares

The glasswares were cleaned by dipping in a solution of 5 % HCl and then washed with detergent in running tap water. The glasswares were then rinsed with double distilled water and dried in the hot air oven at 100°C

before use. Fresh plastic wares such as tarson tubes, petri dishes, microtips, flasks, beakers, PCR tubes of Tarson Company were used in the experiment.

Design of study for subchronic toxicity

Thiamethoxam at two dose levels 2.5% (105 mg) and 5% (210 mg) of MTD (MTD = 4200mg/kg) (Sole *et al.*, 2008) was administered orally daily for 60 and 90 days in adult male rats for subchronic toxicity study. An ameliorative effect of quercetin at dose rate of 50 mg/kg orally was also studied in rats for 60 and 90 days.

Details of each group were as follows:

Group 1: Vehicle control (16 rats): 2% gum acacia suspension in distilled water was given orally at the dose rate of 1ml/200g b.wt. once daily for 60 and 90 days.

Group 2: Thiamethoxam (2.5% of MTD) (16 rats): Thiamethoxam suspension in 2% gum acacia in distilled water was administered orally once daily for 60 and 90 days.

Group 3: Thiamethoxam (5% of MTD) (16 rats): Thiamethoxam suspension in 2% gum acacia in distilled water was administered orally once daily for 60 and 90 days.

Group 4: Quercetin (50 mg/kg) (16 rats): Quercetin suspension in 2% gum acacia in distilled water was administered orally once daily for 60 and 90 days.

Group 5: Thiamethoxam (2.5% of MTD) and Quercetin (50 mg/kg) (16 rats): Quercetin and thiamethoxam suspension in 2% gum acacia in distilled water were administered orally once daily for 60 and 90 days. A gap of 12 hours was maintained between thiamethoxam and quercetin administration.

Group 6: Thiamethoxam (5% of MTD) and Quercetin (50 mg/kg) (16 rats): Quercetin and thiamethoxam suspension in 2% gum acacia in distilled water were administered orally once daily for 60 and 90 days. A gap of 12 hours was maintained between thiamethoxam and quercetin administration.

Preparation of tissue homogenates

Tissue homogenates of testes were prepared in ice-cold PBS (pH 7.4) with tissue homogenizer. The homogenates (5.0%) were prepared under ice cold condition and were centrifuged for 10 min at 5000 rpm. The supernatant was further diluted to 2.5%, 1.25%, 0.625% and 0.15625% conc. using PBS (pH = 7.4) depending upon parameter to be studied in a tissue. A double beam UV-VIS spectrophotometer was used for recording the absorbance of the test samples.

Assessment of oxidative stress and antioxidant parameters in tissues

Oxidative stress parameters such as protein carbonyl content, nitrite and tissue protein was determined as described by the methods of Levine *et al.*, (1990), Sastry *et al.*, (2002) and Lowry *et al.* (1951), respectively. Glutathione -S- transferase activity (GPx), glutathione-S-transferase (GST) and reduced glutathione (GSH) in tissues (antioxidant parameters) were estimated as per the method described by Hafeman *et al.* (1974), Habig *et al.* (1974) and Beutler (1963), respectively.

Histopathological examination

Histopathology of testes was done as described by to Luna (1968). A portion of the specimen (Testes) was fixed in 10% buffered formalin for at least a week period for

histopathological studies. The samples were subjected to overnight washing; the specimen were then dehydrated in ascending grades of alcohol, cleared in benzene and then embedded in paraffin wax to prepare paraffin blocks. Then 5 μ m thick sections were cut with microtome and subjected to further processing and finally stained with hematoxylin and eosin (H&E stain). The slides were observed under microscope for assessment of histopathological changes.

Statistical analysis

Data were expressed as Mean \pm standard error of mean (SEM). Statistical analysis of data was performed using Graph Pad prism 5.03 and Microsoft Excel. Data were analyzed by ANOVA along with Bonferroni multiple comparison post hoc test. A value of ($p < 0.05$) was considered statistically significant (Mead and Curnow, 1982).

RESULTS AND DISCUSSION

Protein carbonyl content

Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on protein carbonyl content level in testes tissue of different treatment groups of both schedules are expressed in nmoles of DNPH/mg protein as presented in Table 1 and Fig. 1. In 60 days treatment schedule, the levels of protein carbonyl content were significantly ($p < 0.05$) higher in 5.0% TMX group as compared to control and quercetin groups. In 90 days treatment schedule, the levels of protein carbonyl content were significantly ($p < 0.05$) higher in 2.5% TMX and 5.0% TMX groups as compared to control and quercetin groups. There was significant decrease in the level of protein carbonyl content in 5% MTD + Qu treated group as compared to 5% TMX in 60 days schedule. The level decreased significantly in 90 days schedule in 2.5% TMX + Qu and 5.0% TMX + Qu groups as compared to 2.5% TMX and 5.0% TMX groups, respectively.

TABLE 1: Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on oxidative stress parameters in testes

Parameters	Days	Values are expressed as Mean \pm SEM (n=8).					
		Control	2.5% TMX	5.0% TMX	Qu (50 mg/kg)	2.5% TMX + Qu	5.0% TMX + Qu
Protein Carbonyl content (nmoles of DNPH/mg protein)	60	2.52 \pm 0.0357	2.61 \pm 0.1766	3.14 \pm 0.1523 ^{ab}	2.21 \pm 0.06132 ^c	2.33 \pm 0.07472 ^c	2.50 \pm 0.1397 ^c
	90	2.63 \pm 0.0344	6.56 \pm 0.4090 ^a	6.72 \pm 0.1910 ^a	3.24 \pm 0.1006 ^{bc}	4.61 \pm 0.176 ^{abcd}	5.42 \pm 0.3392 ^{abcd}
Nitrite nanomole/g tissue	60	250.22 \pm 24.8	373.97 \pm 1.177 ^a	396.89 \pm 1.188 ^a	273.69 \pm 1.068 ^{bc}	328.42 \pm 2.821 ^{abcd}	334.39 \pm 3.678 ^{acd}
	90	280.36 \pm 2.38	390.64 \pm 1.906 ^a	461.61 \pm 3.474 ^{ab}	268.56 \pm 2.705 ^{bc}	301.47 \pm 4.619 ^{abcd}	348.70 \pm 5.922 ^{abcde}
Tissue protein (mg protein /g tissue)	60	189.01 \pm 1.05	103.09 \pm 2.496 ^a	84.91 \pm 1.988 ^{ab}	196.28 \pm 0.3489 ^{abc}	200.59 \pm 1.497 ^{abc}	195.35 \pm 0.6616 ^{bc}
	90	184.78 \pm 0.85	70.95 \pm 3.89 ^a	60.29 \pm 0.83 ^a	160.59 \pm 5.44 ^{abc}	159.92 \pm 5.13 ^{bc}	149.01 \pm 8.09 ^{bc}

a, b, c, d, e ($p < 0.05$) vs. control, 2.5% TMX, 5.0% TMX, Qu and 2.5% TMX + Qu, respectively. 2.5% TMX means 2.5% MTD of thiamethoxam /kg b.wt. (105 mg/kg b.wt orally).

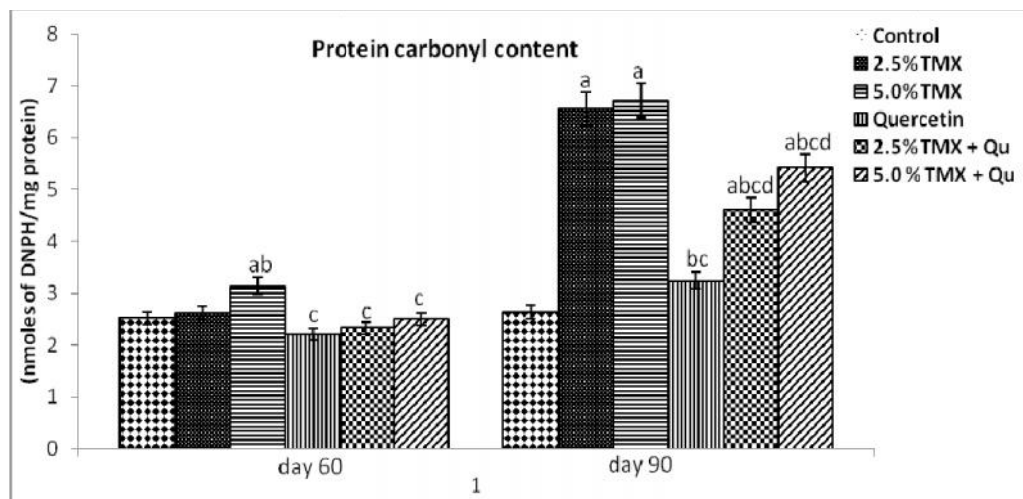


FIGURE 1: Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on testes protein carbonyl content

Nitrite

Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on nitrite level in testes tissue of different treatment groups of both schedules are expressed in nanomol/g tissue as presented in Table 1 and Fig. 2. In 60 and 90 days treatment

schedule, the levels of nitrite were significantly ($p < 0.05$) higher in 2.5% TMX and 5.0% TMX groups as compared to control and quercetin groups. The levels of nitrite decreased significantly in both treatment schedules in 2.5% TMX+Qu and 5.0% TMX +Qu groups, respectively.

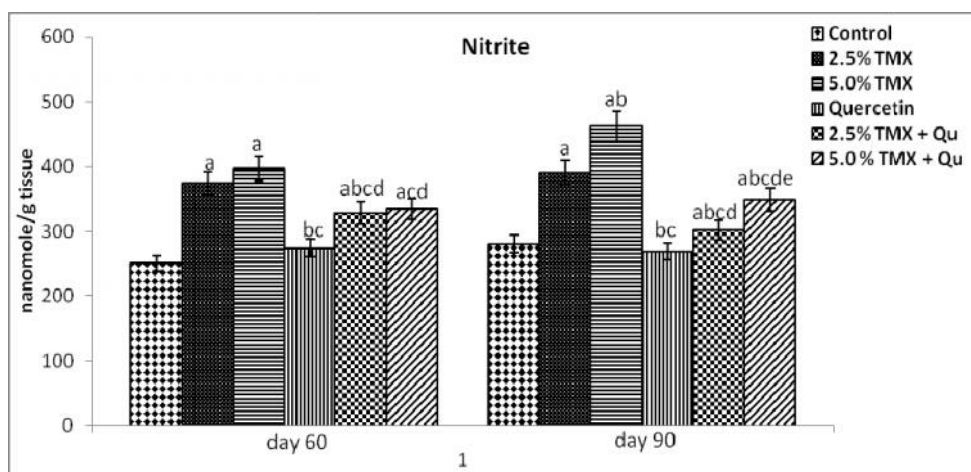


FIGURE 2: Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on testes nitrite.

TABLE 2: Effect of subchronic oral exposure male rats to thiamethoxam, quercetin and their combination on antioxidant parameters in testes.

Parameters	Days	Values are expressed as Mean ± SEM (n=8).					
		Control	2.5% TMX	5.0% TMX	Qu (50 mg/kg)	2.5% TMX + Qu	5.0% TMX + Qu
GPx (U/mg protein)	60	2.01 ± 0.025	0.69 ± 0.047 ^a	0.65 ± 0.039 ^a	1.82 ± 0.134 ^{bc}	1.61 ± 0.03 ^{abc}	1.26 ± 0.034 ^{abcde}
	90	2.35 ± 0.077	1.81 ± 0.587 ^a	5.15 ± 0.308 ^{ab}	2.04 ± 0.041 ^c	2.03 ± 0.056 ^c	1.83 ± 0.106 ^c
GST (µmol/min/mg protein)	60	0.109 ± 0.00069	0.200 ± 0.00495 ^a	0.243 ± 0.00582 ^{ab}	0.105 ± 0.00023 ^{bc}	0.103 ± 0.00075 ^{bc}	0.105 ± 0.00042 ^{bc}
	90	0.111 ± 0.00049	0.295 ± 0.01266 ^a	0.342 ± 0.00483 ^a	0.129 ± 0.00462 ^{bc}	0.129 ± 0.00422 ^{abc}	0.141 ± 0.00817 ^{abc}
GSH (millimol/mg protein)	60	3.24 ± 0.019	0.29 ± 0.054 ^a	0.09 ± 0.023 ^a	3.65 ± 0.048 ^{abc}	2.71 ± 0.043 ^{abcd}	2.21 ± 0.103 ^{abcde}
	90	3.65 ± 0.016	0.39 ± 0.049 ^a	0.11 ± 0.017 ^{ab}	3.84 ± 0.019 ^{abc}	3.38 ± 0.027 ^{abcd}	3.14 ± 0.033 ^{abcde}

a, b, c, d, e (p < 0.05) vs. control, 2.5% TMX, 5.0% TMX, Qu and 2.5% TMX + Qu, respectively. 2.5% TMX means 2.5% MTD of thiamethoxam/kg b.wt. (105 mg/kg b.wt orally).

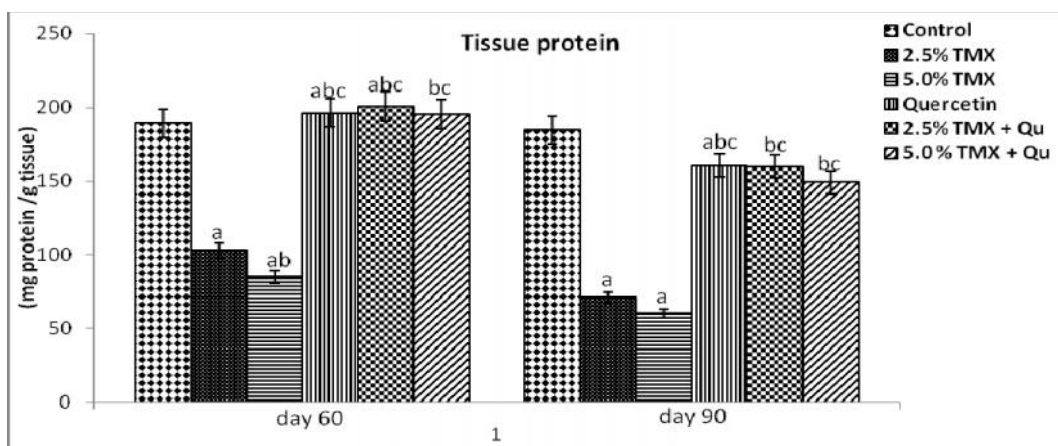


FIGURE 3: Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on testes tissue protein.

Tissue protein

Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on tissue protein level in testes tissue of different treatment groups of both treatment schedules are expressed in mg protein /g tissue as presented in Table 1 and Fig. 3. In 60 and 90 days schedules, the levels of tissue protein were significantly (p < 0.05) lower in 2.5% TMX and 5.0% TMX groups as compared to control and quercetin groups. There was significant increase in the level of tissue protein in 2.5% TMX + Qu and 5.0% TMX + Qu groups as compared to 2.5% TMX and 5.0% TMX groups, respectively.,

Glutathione peroxidase (GPx)

Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on glutathione peroxidase level in testes tissue of different treatment groups of both schedules are expressed in Unit/mg protein as presented in Table 2 and Fig. 4. In 60 days treatment schedule, the level of GPx activity was significantly (p < 0.05) lower in 2.5% TMX and 5.0% TMX groups, whereas at 90 days treatment schedule, significant decrease was observed in 2.5% TMX group while increase was observed in 5.0% TMX group as compared to control and quercetin group. There was

significant increase in glutathione peroxidase level in 2.5% TMX + Qu and 5.0% TMX + Qu groups as

compared 2.5% TMX and 5.0% TMX groups, respectively in 60 days schedule.

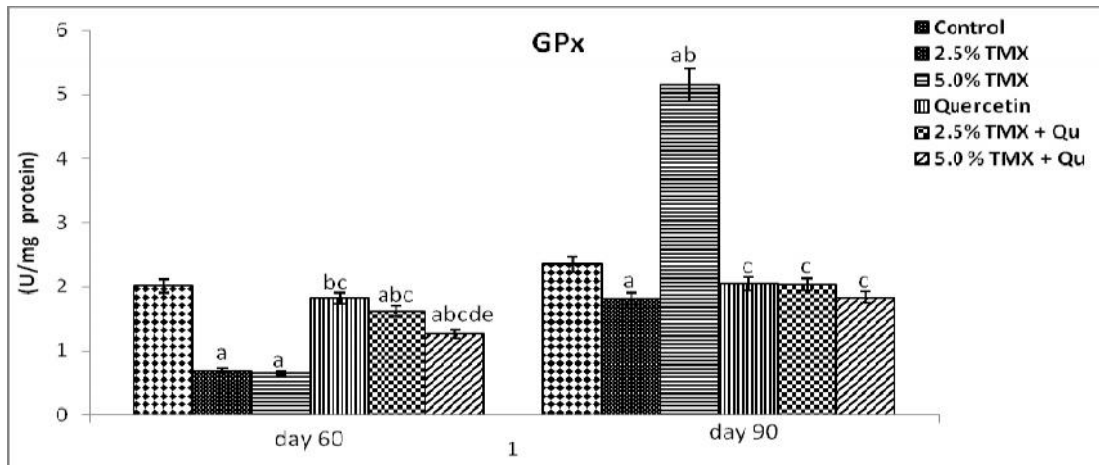


FIGURE 4: Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on testes glutathione peroxidase.

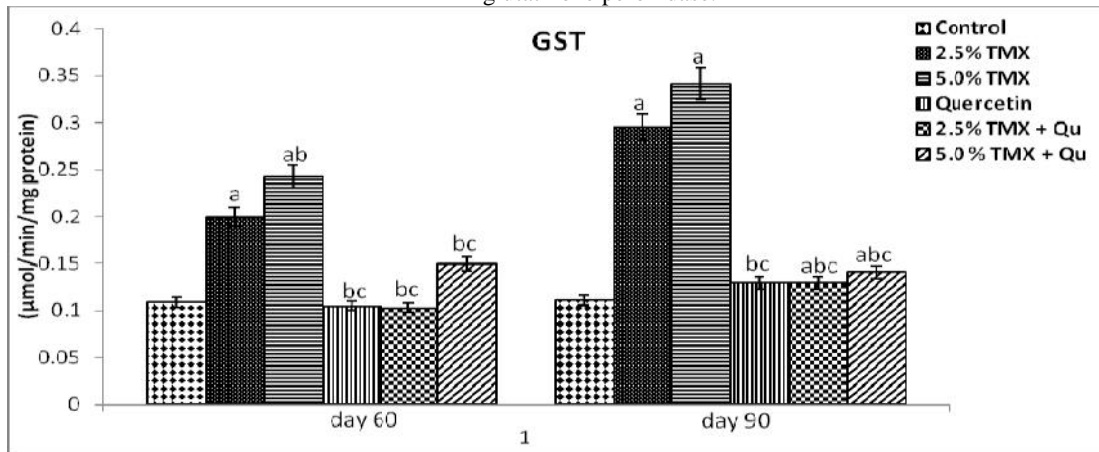


FIGURE 5: Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on testes glutathione -s-transferase.

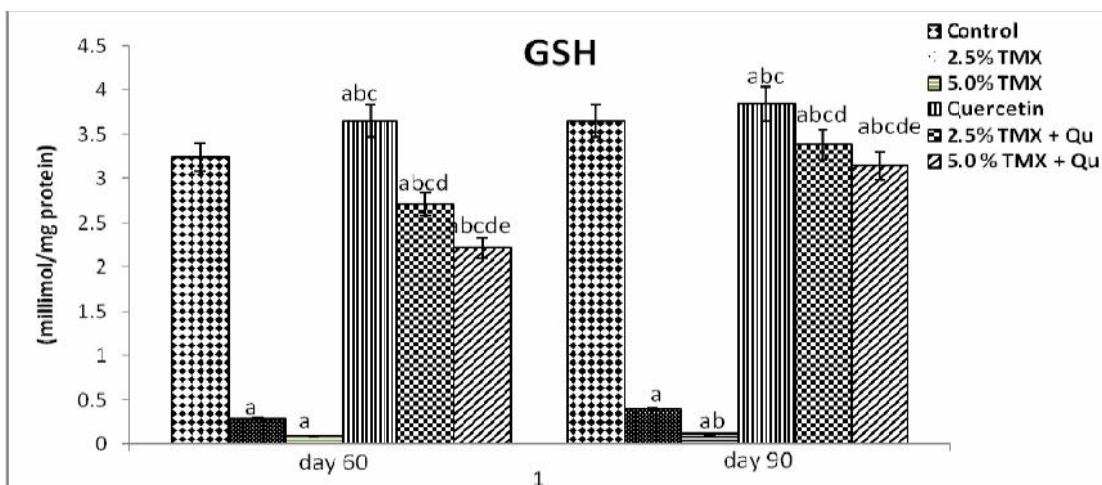


FIGURE 6: Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on testes reduced glutathione.

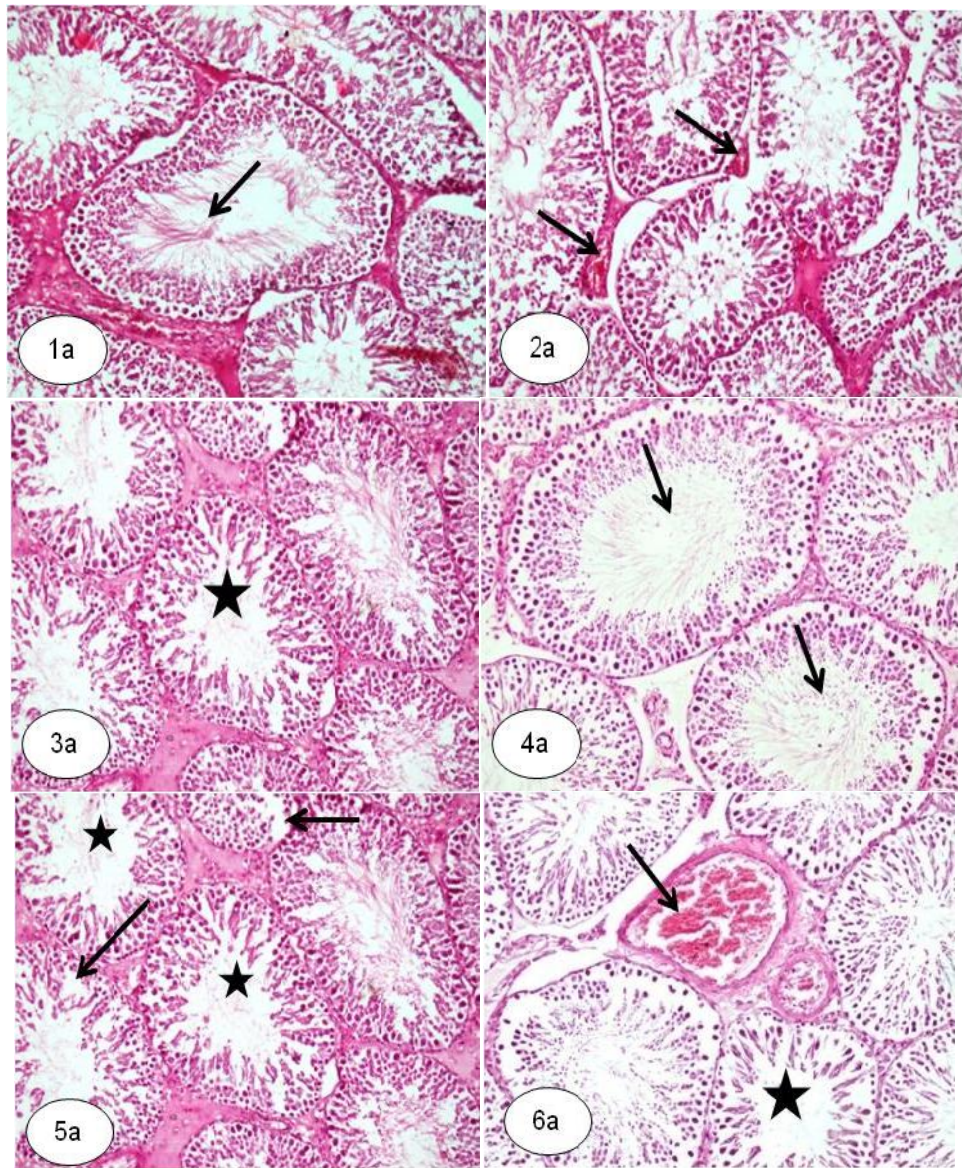
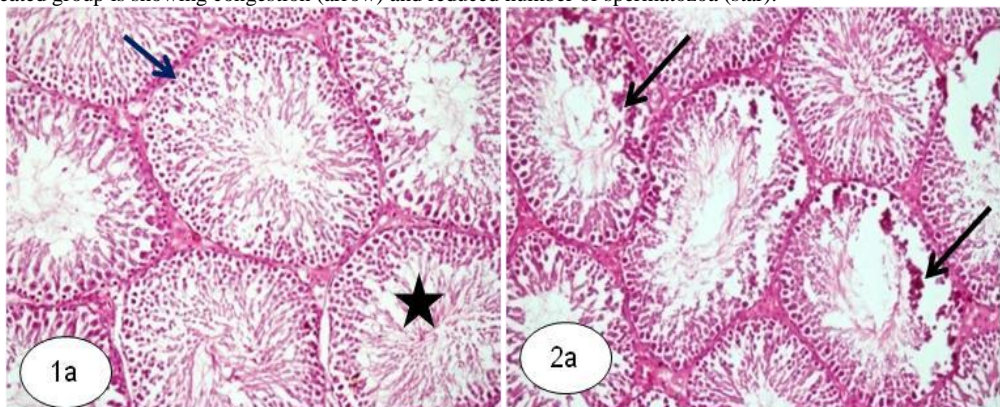


FIGURE 7: Representative photomicrographs (H & E $\times 400$) of testes of male rats (1a) - vehicle, (2a)- 2.5% TMX, (3a) – 5.0% TMX, (4a) - Qu, (5a)- 2.5% TMX+ Qu- and (6a)- 5.0% TMX + Qu-treated group after 60 days schedule. Vehicle-treated group showing normal testes histology with intact germinal epithelium and lumen filled with normal spermatozoa. Lower dose 2.5% TMX-treated group is showing mild congestion in inter tubular capillaries of testes. Higher dose 5.0% TMX-treated group is showing mild sloughing of germinal epithelium and reduced number of spermatozoa in lumen of some of seminiferous tubules. Qu-treated group is showing normal seminiferous tubules filled with normal spermatozoa in lumen. 2.5% TMX+ Qu-treated group is showing mild sloughing of germinal epithelium (arrow) and reduced number of spermatozoa in lumen of some seminiferous tubules (star). 5.0% TMX+ Qu-treated group is showing congestion (arrow) and reduced number of spermatozoa (star).



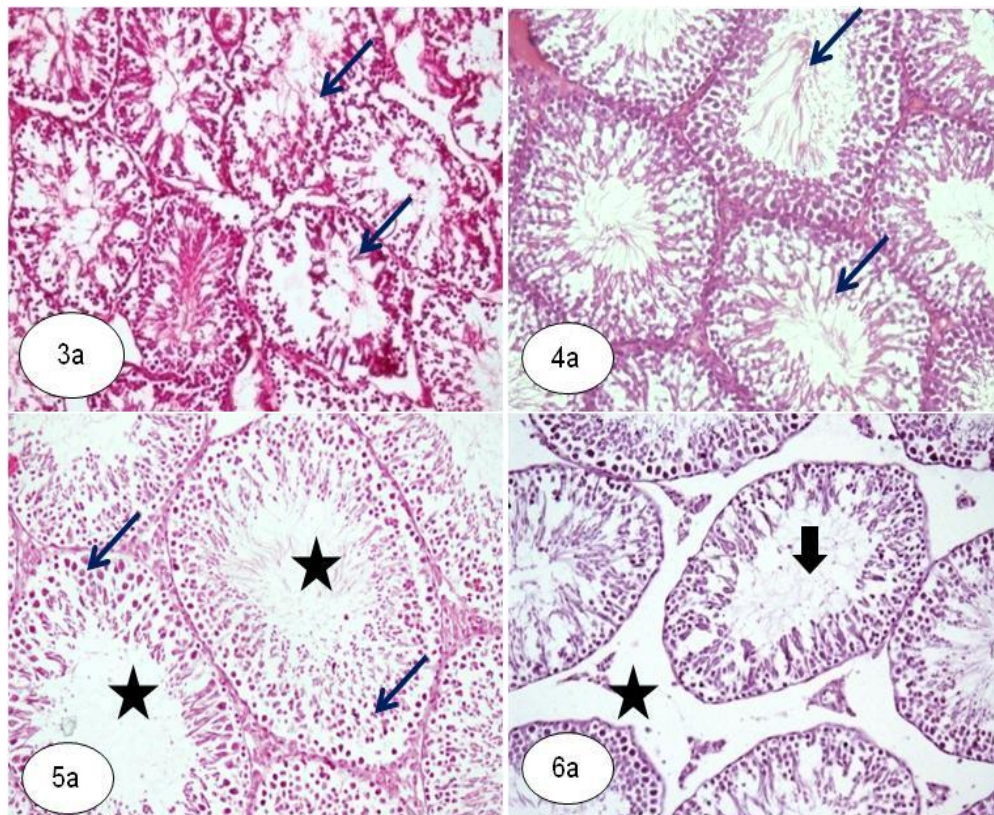


FIGURE 8: Representative photomicrographs (H & E $\times 400$) of testes of male rats (1a) - vehicle, (2a)- 2.5% TMX, (3a) – 5.0% TMX, (4a) - Qu, (5a)- 2.5% TMX+ Qu- and (6a)- 5.0% TMX + Qu-treated group after 90 days schedule. Vehicle-treated group showing normal histology with intact germinal epithelium (arrow) and lumen filled with spermatozoa (star). Lower dose 2.5% TMX-treated group is showing mild degeneration of seminiferous tubules with sloughing of germinal epithelium (arrow). Higher dose 5.0% TMX-treated group is showing degeneration of seminiferous tubules with sloughing of germinal epithelium. (Arrow). Qu-treated group is showing normal histological structure of seminiferous tubules filled with spermatozoa (arrow). 2.5% TMX+ Qu-treated group is showing detachment of spermatogonia (arrow) and reduced number of spermatozoa in lumen of seminiferous tubules (star). 5.0% TMX+ Qu-treated group is showing widened interstitial space (star), detachment of germinal epithelium in focal areas and reduced number of spermatozoa in lumen of seminiferous tubules (arrow).

Glutathione -S- transferase (GST)

Effect of subchronic oral exposure of male rats to thiamethoxam, quercetin and their combination on glutathione-S-transferase level in testes tissue of different treatment groups of both schedules are expressed in $\mu\text{mol}/\text{min}/\text{mg}$ protein as presented in Table 2 and Fig. 5. In 60 and 90 days treatment schedules, the level of GST was significantly ($p < 0.05$) increased in 2.5% TMX and 5% TMX groups as compared to control and quercetin group. There was significant decrease in GST level in 2.5% TMX + Qu and 5.0% TMX + Qu groups as compared to 2.5% TMX and 5.0% TMX groups, respectively in both treatment schedules.

Reduced glutathione (GSH)

Effect of subchronic oral exposure to thiamethoxam, quercetin and their combination on reduced glutathione level in testes tissue of different treatment groups of both schedules are expressed in millimol/mg protein as presented in Table 2 and Fig. 6. In 60 and 90 days treatment schedules, the level of GSH was significantly ($p < 0.05$) lower in 2.5% TMX and 5% TMX groups as compared to control and quercetin group. There was significant increase in GSH level in 2.5% TMX + Qu and 5% TMX + Qu groups as compared to 2.5% TMX and 5% TMX groups, respectively.

Histopathological lesions

Histopathological lesions in 60 and 90 days in testes of control and treatment groups are presented in Fig.7 and 8. Control group showed normal histology with intact germinal epithelium and lumen filled with normal spermatozoa at both treatment schedules. Histopathological investigations of 2.5% TMX group showed mild congestion in inter tubular capillaries in 60 days treatment schedule, while mild degeneration of seminiferous tubules with sloughing of germinal epithelium were observed in 90 days schedule. Mild congestion in inter tubular capillaries were observed in 5.0% TMX group in 60 days schedule, while degeneration of seminiferous tubules with sloughing of germinal epithelium were observed in 90 days schedule. Rats in Qu group showed normal histology with intact germinal epithelium and lumen filled with normal spermatozoa at both schedules. Quercetin co-treatment group showed mild sloughing of germinal epithelium and reduced number of spermatozoa in lumen of some seminiferous tubules in 2.5% TMX + Qu group in 60 days schedule, while detachment of spermatogonia and reduced number of spermatozoa in lumen of seminiferous tubules were observed in 90 days schedule. Congestion and reduced number of spermatozoa were observed in 5.0% TMX + Qu

group in 60 days schedule, whereas widened interstitial space, detachment of germinal epithelium in focal areas and reduced number of spermatozoa in lumen of seminiferous tubules were observed in 90 days schedules, respectively.

Thiamethoxam is a highly effective systemic and contact insecticide with relatively large oral LD50 in albino rats (1563 mg/kg b.wt.) indicating low acute mammalian toxicity (Shalaby *et al.*, 2010). Neonicotinoid insecticides represent the fastest growing class of insecticides introduced to the market since the launch of pyrethroids (Nauen *et al.*, 2001). The current market share of this class is well above 600 million Euros per year, including imidacloprid as the biggest selling insecticide worldwide (Jemec *et al.* 2007). Another neonicotinoid commercialized since the introduction of imidacloprid is thiamethoxam (Tomizawa and Casida, 2003).

Exposure to pesticides has been associated with many harmful effects including hormonal disorders, mutagenic and carcinogenic events due to cellular apoptosis and lipid peroxidation in humans and animals (Dikshith, 1991). Reactive oxygen species (ROS) generated due to exposure to pesticides can cause tissue damage by triggering several oxidative mechanisms and lipid peroxidation (Kanbur *et al.*, 2008). ROS are mainly composed of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen (O^-). The ROS can attack DNA, producing a distinctive pattern of DNA alterations (Kovacic and Somanathan, 2008).

The effect of toxicity of thiamethoxam at 2.5% and 5.0% MTD alone and in combination with quercetin (Qu, 50 mg/kg orally) was studied at 60 and 90 days treatment schedules on oxidative stress, nitrosative stress, antioxidant parameters and histopathology in the testes. Oxidative stress parameters such protein carbonyl content (PCC), nitric oxide (NO) concentration and tissue protein; antioxidant parameters such as glutathione peroxidase (GPx), glutathione-S-transferase and reduced glutathione (GSH). The ameliorative potential of quercetin (50mg/kg orally) on above parameters was observed in male rats in this study.

There was increase in protein carbonyl content in the testes in 5.0% TMX group in 60 days schedule and in both the groups in 90 days schedule indicating oxidative stress. Amelioration in the testes was observed in 5.0% TMX + Qu group in 60days schedule and in both groups in 90 days schedule. Exposure of male rats to TMX in subacute toxicity study significantly increased protein carbonyl content levels in liver, testes and epididymis (Nigam, 2015). There was increase in the levels of protein carbonyl content levels in liver, kidney, uterus, ovary and adrenal gland in female rats exposed to thiamethoxam in subacute toxicity study (Singh, 2016).

Nitrite level in the testes significantly increased in both groups and in both schedules compared to control and quercetin group. Amelioration by quercetin in the testes was observed in both the groups in both schedules compared to 2.5% TMX and 5.0% TMX groups, respectively.

Nitrite has been considered as one of the inflammatory mediator playing a significant role in the pathogenesis of various diseases in nature (Kolios *et al.*, 2004). Nitrite is a highly reactive molecule that reacts freely with active

oxygen species to generate a compound such as peroxynitrite that is detrimental to animal cells due to apoptotic potential of this compound. Peroxynitrite lead to the causation of mutagenicity, teratogenicity and carcinogenicity in biological system (Pryor and Squadrito, 1995). Plasma protein level is a significant indicator of health condition, metabolic and production features of the organism because of numerous roles in the pathophysiology. Therefore, plasma proteins have an exceptional significance in homeostasis as they play important roles in maintenance of colloid osmotic pressure.

The tissue protein level in the testes in this study decreased significantly in both the groups in both schedules compared to control and quercetin group. Amelioration in testes was observed in both the groups and in both schedules as compared to 2.5% TMX and 5.0% TMX groups.

Decreased protein levels seen may be attributed to stress mediated immobilization of proteins to fulfil an increased demand for energy for the detoxification process (Jenkins and Smith, 2003). Decreased protein level in tissues may be due to excessive loss through nephrosis (Rahman *et al.*, 1990) as kidney is affected in the present study.

In testes there was, significant decrease in values of glutathione peroxidase (GPx) was observed except in 5.0% TMX group in 90 days schedule. There was amelioration in the level of GPx in the testes in both groups in 60 days schedule. Glutathione peroxidase (GPx) is an enzyme that removes hydrogen peroxide (H_2O_2) generated by superoxide dismutase (SOD) in cytosol and mitochondria by oxidizing GSH to GSSG. This enzyme prevents generation of hydroxyl radicals and protects cellular constituents from oxidative damage (Scoat *et al.*, 1991). Glutathione peroxidase (GPx) enzymes constitute a mutually supportive team of defense against reactive oxygen species protecting the cells against oxidative stress (Venukumar and Latha, 2002).

The level of glutathione- S-transferase (GST) in the testes increased significantly in 2.5% TMX and 5.0% TMX groups as compared to control and quercetin group in both treatment schedules. Amelioration by quercetin in the level of GST in the testes was observed in both the groups in both treatment schedules as compared to 2.5% TMX and 5.0% TMX groups, respectively. Glutathione S-transferases (GSTs) previously known as ligandins and consists of three super families: the cytosolic, mitochondrial, and microsomal, are involved in detoxification of xenobiotic compounds and in the biosynthesis of important metabolites. Glutathione S-transferases comprise a family of eukaryotic and prokaryotic phase II metabolic isozymes best known for their ability to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. The Glutathione-S-transferases family consists of three super families: the cytosolic, mitochondrial, and microsomal (Rahman *et al.*, 1990; Jenkins and Smith, 2003).

The level of reduced glutathione (GSH) decreased significantly in 2.5% TMX and 5.0% TMX groups as compared to control and quercetin group in both treatment schedules in testes. Amelioration by quercetin in these

tissues was observed in both treatment groups of both schedules. GSH is cell's natural antioxidant which destroys free radical formed in cells. Free radicals have become an attractive means to explain the toxicity of numerous xenobiotics (e.g. pesticides) and some of these free radicals interact with various tissue components, resulting in dysfunction. Oxidative damage due to excessive production of reactive oxygen species (ROS) has been associated with defective organs dysfunction and the inhibition of enzymes involved in free radical removal led to the accumulation of H₂O₂, which promoted lipid peroxidation and modulation of DNA, altered gene expression and cell death. GSH is known to play an important role in scavenging ROS (Beutler *et al.*, 1963) Histopathological investigations of the testes demonstrated that control and quercetin treated animals showed normal testes histology with intact germinal epithelium and lumen filled with normal spermatozoa. Animals in 2.5% TMX group in both schedules indicated mild congestion in inter tubular capillaries of testes and cloudy swelling changes. Testes of rats in 5.0% TMX group in both schedules showed mild sloughing of germinal epithelium and reduced number of spermatozoa in lumen of some of seminiferous tubules. Observations in animals in 2.5% TMX + Qu co-treated group at both treatment schedules indicated mild sloughing of germinal epithelium, reduced number of spermatozoa in lumen of some seminiferous tubules and detachment of spermatogonia. Observations in animals in 5.0% TMX + Qu co-treated group at both treatment schedules indicated congestion, widened interstitial space, detachment of germinal epithelium in focal areas and reduced number of spermatozoa in lumen of seminiferous tubules. In some earlier studies on pesticides especially imidacloprid, it has been found that histopathology of testes of rat showed reduced or lack of spermatogenesis in few tubules and interstitial edema after 28 days exposure (Lonare *et al.*, 2015). Quercetin co-treatment in thiamethoxam treatment groups provided improvement in histopathological changes of testes by increasing the size of germinal epithelium and promoting spermatogenic cycle.

Authors' Contributions Dr Vinod Kumar designed the study. Dr Mohammed Shaibu Auwal conducted the experiment and performed statistical analysis. All authors read and approved the final manuscript.

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Competing Interests The authors declare that they have no competing interest.

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