Gene expression endpoints following subchronic thiamethoxam exposure in adult male rabbits

Abstract

Aim: to investigate the sub chronic toxicity of TMX on some parameters of reproductive performance in adult male rabbits including gene expression of LDH-C4, FSH*β* and LH*β and* GnRHR.

Method: 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 $mgkg^{-1}$ ~~b.wt~~ body weight for 90 days. The second group was served as control.

Result: Obtained results showed that TMX increased the relative weight of some reproductive organs including testis and prostate. 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. In addition, LDH-C4, FSH*β* and LH*β and* GnRHR genes were down regulated in TMX treated group.

Conclusion: administration of TMXfor 90 days in male rabbits induced a noticeable adverse effect on serum testosterone level and down regulated genes related to male rabbit reproductive performance.

Keywords: Thiamethoxam, Neonicotinoids, gene expression, GnRHR, FSH, LH, LDH-C4, hormones, testis, sub chronic toxicity.

INTRODUCTION

Neonicotinoids is a new class of insecticides developed in ~~pastmillenniumpast~~ millennium in 1990 and gain a popularity in the beginning of 2003. Their marker

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has been grown up with global annual sales of $ 3.5 billion[37]. Insects showed lower resistance to neonicotinoids, in contrary with organo-phosphorus compounds and pyrethroids. Moreover, they have lower toxicity risk to non-target species. This class of insecticide is used predominantly in seed cropping but recently has been introduced into the field of veterinary medicine to combat with fleas and flies. Neonicotinoids are regarded as nAChRs (nicotinic acetyl choline receptor) agonists leading to spontaneously activation of theses receptors then paralysis and death of insects. Thiamethoxam, clothianidin, thiacloprid, acetamiprid, dinotefuran and nitenpyram are the most the most popular insecticides in this class [10, 35, 36].

Toxicological profile showed that, thiamethoxam (TMX) is rapidly absorbed from the gut and perfused throughout the body tissues [6]. It is metabolized by hepatic microsomal CYPs into desmethyl TMX and clothianidin that is further metabolized into desmethyl clothianidinwhich act as inducible nitric oxidase synthase inhibitor. Desmethyl TMX and desmethyl clothianidinare hepatotoxic and hepato-carcinogenic [18, 19]. Furthermore, another study revealed formation of formaldehyde and m-methylol intermediates which are considered as an alternative mechanism for hepatotoxicity/hepato-carcinogenecity [32]. Furthermore, in another pharmacokinetic study TMX metabolites were found in brain of mice administrated TMX in dose 20 mg/kg intraperitoneally [16].

In addition, the reported materials indicated thiamethoxam interfered with reproductive function of laboratory animals. Rats received TMX at dose 100mg/kg showed histopathological alterations in testicular tissue including depletion and hyalinization of germinal epithelial cells of seminiferous tubules[14]. Moreover, metabolite of thiamethoxam, clothianidin has adverse effect on reproductive performance. Bal, R et., demonstrated that clothianidin increased significantly level thiobarbituric acid-reactive substances in testicular tissue. Also clothianidin insignificantly increased testicular apoptotic index in a dose dependent manner [3].

Gene expression is an important endpoint in assessing toxicity of chemical substances and insecticides. Reproductive process in mammals is controlled by the hypothalamic-pituitary-gonadal axis. gonadotropin-releasing hormone (GnRH) is

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secreted from the hypothalamus and regulates both expression and secretion of luteinizing hormone (LH), and follicle-stimulating hormone (FSH) from the anterior pituitary gland[30]. This cycle is mediated by GnRH receptor located on the surface of pituitary gland[31]. LH and FSH in turn stimulate steroidogenesis and gametogenesis in the gonads [9, 34].Lactate dehydrogenase c4 is isoenzyme is found in male and female germs. It is essential for glycolysis process for ATP production, which is responsible for capacitation and motility of spermatozoa. disruption or deficit in this enzyme or his expression impairs male fertility, but not in females[12]. It was reported that activity of LDH-C4in knockout sperm is 18% only of his activity in normal sperm [12, 26].Here we study effect of thiamethoxam on gene expression of reproductive process related genes as FSHβ, LHβ and GnRHR in pituitary gland and LDH-C4 gene in testicular tissue.

Materials and methods

Description of the study area

The study was conducted …….

Chemicals and reagents

A commercial product of thiamethoxam25% (TMX 25%) was purchased from a local pesticide market under a trade name Actara®, Syngenta co., Canada.Direct-zol™ RNA miniprep extraction kit, Hisenscript™RH (-) cDNA synthesis kit and 5x HOT FIREPol®EvaGreen®qPCR Mix Plus ROX kit were purchased from Zymo Research Corp (USA), iNtRON Biotechnology (Korea) and SolisBioDyne (EU), respectively. ELISA kits used to measure concentration of serum FSH, and LH were obtained from CUSABIO®, China. Testosterone ELISA kit was purchased from Immunotech Beckman Coulter Company, USA. All other reagents are of good analytical and diagnostic value or the best available pharmaceutical grade.

Preparation of TMX

About 10g Actara®25% was dissolved in 30 ml distilled water as stock solution to be orally administrated for each rabbit at a dose of 3mlKg^{-1}b.w which equivalent 250mg Kg^{-1}b.w.

Animals and experimental design

The present experiment was carried out on 20 adult male *Chinchilla* rabbits 6.0-6.5 ~~months~~month's old and weighing 2.5-2.8 kg. Animals were obtained from

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a commercial rabbit farm. They were housed in metallic batteries in a room with a temperature of 24±0.5 °C. Rabbits were fed on a balanced commercial diet with protein not less than 19% and provided with clean water *ad-libitum*. The ration composed of bran, yellow corn, hay, molasses, and soybean 44%. Animals were kept under supervision without any type of treatment for 2 weeks prior to the experiment for any abnormal signs and to ensure normal behavior and growth. Animals were divided randomly into groups with eight rabbits in each group. Treated group was administrated thiamethoxam orally by oral gavage at a dose of 250 $mgkg^{-1}$b. w for 90 days. Controlled group was administrated equivalent volume of distilled water. Oral LD50 of thiamethoxam in rabbits is 5000 ppm as indicated by material safety data sheet prepared by Syngenta, ~~Canada Inc~~Canadian. So, the selected dose of thiamethoxam was approximately $\frac{1}{20}$ LD_{50}in rabbits.

After the end of the experiment blood samples were collected from ear vein in a tube without anticoagulant and ~~centrifugated~~centrifuged at 3000 RPM for 10 minutes to separate serum for assessment of FSH, LH and testosterone hormones. After blood collection, animals were weighted and euthanized. Reproductive organs were dissected and weighted to calculate their relative weight according to the formula described by Bal et al.[3]. right testis and pituitary gland were rapidly collected and preserved at -80 c until the assessment of gene expression.

Assessment of serum FSH, LH and testosterone hormones

The concentration of serum FSH and LH were assessed using specific sandwich enzyme linked immunoassay (ELISA). In short, our sample and serially diluted standard solution was added in ELISA plate and incubated. After that 100 ul biotin-antibody substance were added to each well to bind hormones the plate was washed. Then, 100 ul horseradish peroxidase avidin reagent were added and plate was incubated at 37 ^{o} c. after 1 hour plate was washed and 90 ul substrate was added to each well and incubated for 15 minutes. Finally, The reaction with stopped using stop solution included in the kit. The concentration of serum hormones were measured in ELISA apparatus at 450 nm.

Serum testosterone concentration was measured using competitive ELISA. 10 ul of sample and standard were pipetted to plate with 50 ul of testosterone enzyme in each well, mixed well and incubated. Then 50 ul testosterone biotin reagent was added and after 1 hour incubation at room temperature, the plate was washed. Then, 100 ul substrate solution was added and plate incubated for 15 minutes at room temperature. Finally, reaction was stopped by 50 ul stop solution. Absorbance was measured at 450nm.

Real-time PCR of pituitary FSH*β*, LH*β* and GnRHR genes and testicular LDH-C4 gene

The expression of mRNA of pituitary FSH-*β*, LH-*β* and GHRHR genes and testicular LDH-C4 was assessed by using real time PCR (RT-PCR). RNA was firstly extracted from pituitary and testicular samples using Direct-zol^{tm} RNA miniprep extraction kit (USA). For this purpose, 10 g from pituitary or 25 grams from testis were homogenized with triazole reagent included in the kit with a tissue homogenizer. Then the samples were ~~centrifugated~~centrifuged to remove debris and the supernatant was transferred to an RNA-ase free tube. 600 ul ethanol were mixed thoroughly with the supernatant then the mixture was transferred to special column to obtain pure mRNA. To elute RNA from the column, 50 ul of DNase/RNase-free water were added to the column and ~~centrifugated~~centrifuged at 14000 x g for 30 seconds. The quality of isolated RNA was detected on 2% agarose and visualized by gel documentation system and its concentration will determined by U.V spectrophotometer.

Secondly, extracted RNA was RNA was reversely ~~transcripted~~transcript into cDNA by using Hisenscript^{tm} RH [-] cDNA synthesis Kit (Korea). 3 ml RNA template were mixed well with 1 ml reverse transcriptase (RT) enzyme in 0.2 ml PCR tube. Reverse transcription was performed in the PCR at 50 c for 30 seconds followed by another cycle of RTase inactivation at 85 c for 10 minutes. Conventional PCR was used to check quality of the prepared cDNA using GAPDH primer and the following PCR condition: initial denaturation at 94^{o}c for 5 minutes, followed by 35 cycles of 95^{o}c for 1 minute for denaturation, annealing temperature was 60^{o}c for 30 seconds, extension at 72^{o}c for 1 minutes, final extension at 72^{o}c for 10 minutes, and the samples were held at 4^{o}c (reference).

Finally Real-time PCR was performed using the 5x HOT FIREPOL EvaGreenqPCR Mix ~~Plus~~plus (SolisBioDyne, EU). A 20 uL reaction for each examined gene was prepared with 4 ul– 5x hot firepol evagreenqPCR– Mix Plus

(SolisBioDyne, EU), 0.5 ul of each primer (forward and reverse), 3 ul of RNA ~~template ,and~~template, and 11 ul of water PCR grade. Primer sequences and qPCR cycling conditions used are described in table 1.The primers were designed online on http://perlprimer.sourceforge.net and synthesized by LGC Company, USA. GAPDH was used as internal control gene for normalizing mRNA levels of the target genes. it's primer sequence was according to Wei, S et al., 2012 [38]**.** Stratagene MX3005P real time PCR machine was used to perform the reaction. The results of gene expression including threshold cycle (Ct) value were determined using Stratagene MX3005P installed in the RT PCR machine, Where CT is the threshold cycle indicating the fractional cycle number at which the amount of amplified LDHC4, FSH-B, LH-B and GnRHR reached threshold.Ct of the target genes was normalized with Ct of a reference gene in rabbit, in our experiment the selected reference gene is glyceraldehyde-3-phosphate dehydrogenase (GAPDH). the interpretation of gene expression results was carried out according to $2^{-\Delta\Delta Ct}$ method described by Yuan et al. (2006)[40].

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Table 1: Primer sequences used for qPCR for LDHC4, FSH*β*, LH*β* GnRHR and GAPDH (housekeeping) genes.

Gene Abb.	Primer	Annealing temperature	Amplicon size (pb)
LDH-C4	F-GCTGATGAACTCGCCCTTGT R-GACCAGAGCAAGGCGACC	60^{O}C	198
FSH*β*	F-TGGAGAAAGAGGAGTGCCG R-CTTCTGGATGTTGGGCCTTG	60^{O}C	113
LH*β*	F-GCATGGTGCGGGTGCTG R-CGCCAGGTGGACAGCC	60^{O}C	108
GnRHR	F-AACTCCAGAAGTGGACTCAGAA R-CATCCAGTGGCATGACAATCAG	60^{O}C	120
GAPDH*	F-GGTCGGAGTGAACGGATT R-CTCGCTCCTGGAAGATGG	60^{O}C	227

*Housekeeping gene

Statistical analysis

Collected data was analyzed using SPSS software (version 21.0; SPSS, Inc.). Independent t-test was used to determine the significance of differences between the controlled group and thiamethoxam treated group. Results were statistically significant if values $P<0.05$. Data was expressed as mean ± standard error of mean (SEM).

RESULTS

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Relative reproductive organ weight

As shown in fig. (1), significant increases were observed in the relative weights of testis, epididymis, prostate, and seminal vesicle of the treated group when compared with control group ($p<0.05$).

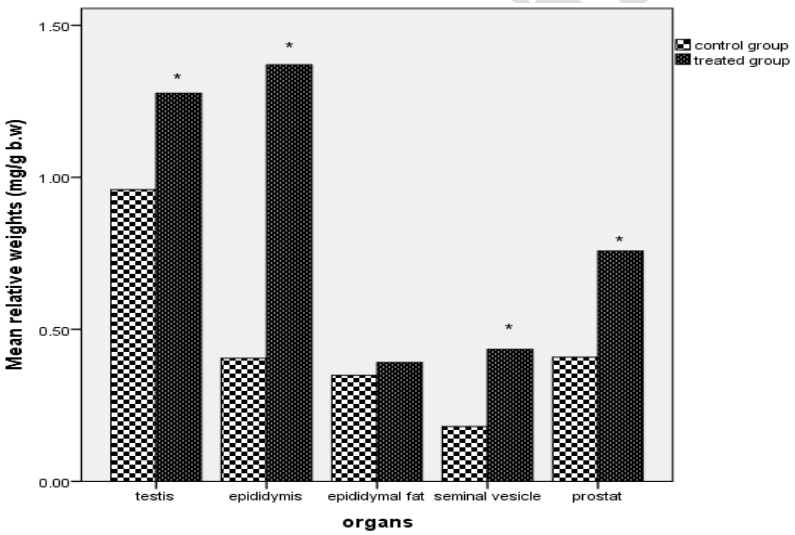

Figure 1: Relative weights of reproductive organs. The values indicated by asterisk are statically significant ($p<0.05$) between groups.

Hormonal analysis

The obtained data in table (2) showed that there was significant increase in serum testosterone level in the treated group *(p<0.05)* while FSH and LH levels did not exhibit significant alterations.

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Table 2: Effect of TMX on serum testosterone, FSH and LH levels.

Groups	Testosterone (ng/ml)	FSH (mlU/ml)	LH (mlU/ml)
Control	1.40 ± 0.19	2.31 ± 1.2	1.73 ± 0.30
Treated	3.16 ± 0.61*	2.21 ± 0.56	1.06 ± 0.48

Results were expressed as mean ± SEM. (n=8).
**p<0.05.*

Quantitative analysis of testicular mRNA expression of LDHc4 (testis-specific gene) and pituitary gland mRNA expression of FSH, LH and GnRHR:

RNA was extracted and the quality and the integrity of total RNA were assessed by inspection of ribosomal RNA band (18s and 28s) in ethidium bromide stained 1.5% agarose gel which were visualized by ultraviolet (UV) light as shown in fig. (2).

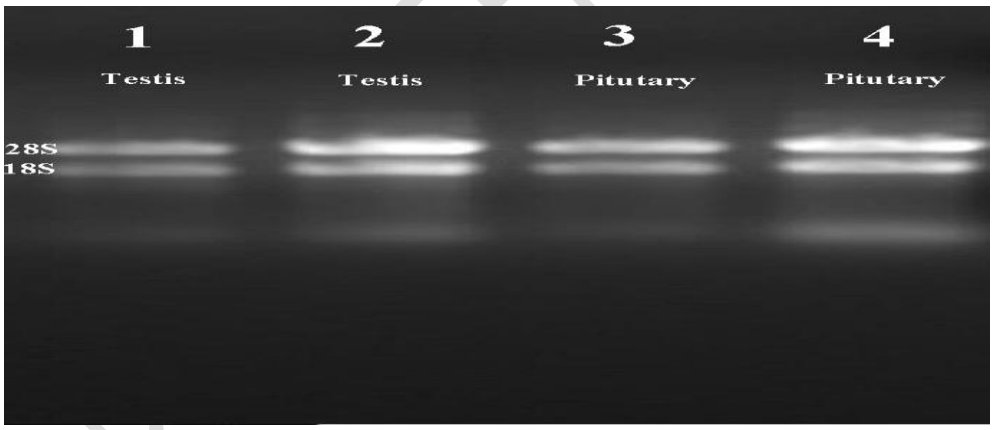

Figure2: Representative image of electrophoresis of total RNA in agarose gel. Lane 1: Testicular RNA of control group, lane 2: Testicular RNA of treated group, lane 3: Pitutary RNA of control group, lane 4: Pitutary RNA of treated group.

The quality of cDNA was assessed using GAPDH primer using conventional PCR and the results were visualized on ethidium bromide-stained agarose.

Table (3) showed that TMX treatment resulted in down regulationof all evaluated genes, LDH-C4, FSH*β*, LH*β* and GnRHR by 0.9, 0.63, 0.64 and 0.57 folds respectively.

Table3: Effect of TMX on gene expression of testicular LDH-C4 and pituitary FSH*β*, LH*β* and GnRHR genes.

Gene name	Gene symbol	Average fold change in expression ($2^{-\Delta\Delta ct}$)	Interpretation
Lactate dehydrogenase c4 isoenzyme	LDH-C4	0.92	Down regulated
Follicular stimulating hormone *βsubunit*	FSH*β*	0.63	Down regulated
Luteinizing hormone *βsubunit*	LH*β*	0.64	Down regulated
Gonadotrophin releasing hormone receptors	GnRHR	0.57	Down regulated

Discussion

Thiamethoxam (TMX) is a novel neonicotinoid insecticide has the potential to induce impairment of many physiological parameters and histological structures in albino rats [29]. 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 [11, 20, 25]. NEOs were classified by EPA as toxicity class II and/or class III agents [7]. The results presented in the current study demonstrate that sub chronic exposure to TMX in adult male rabbits caused negative effects on their reproductive performance.

The statically significant and insignificant increase in relative reproductive organ weights could be explained by the increased testosterone level in TMX treated group because testosterone is the major regulator of normal growth of reproductive organs and body weight [22]. However, this is opposite to the results obtained by Bal et al.after male rats exposed chronically to Imidacloprid [2, 4]. They found significant decrease in relative organ weights in the treated groups with marked

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reduction of body weight and explained this by the significant decrease in serum testosterone level. In the same context, the effects of CLO on the reproductive system organ and body weights of adult male mice were the same, with a reduction in serum testosterone level but insignificantly [3]. This discrepancy can be explained by the species difference effect of TMX.

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 elevation in testosterone concentration is not related to the insecticide effect on pituitary gland and originates directly from testis. This understanding may be attributable to effect of thiamethoxam on rabbit testicular leydig cells because TMX considered as pro carcinogenic chemical substance. Metabolites of TMX as desmethyl TMX and desmetyl clothianidin are very toxic and considered as carcinogenic substances. Therefore, Green et al. reported TMX at a dose of 500mg/kg in mice increased hepatic cell replication and induced tumor. Moreover, Formaldehyde and N-methylol intermediates generated during TMX metabolism are considered as an alternative mechanism of TMX hepatocarcinogenicity in mice.

LDH gene is coding for lactate dehydrogenase proteins that are distributed in tissue and cell-type specific patterns. Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate to lactate with the oxidation of NADH to $NAD^+$with ATP production [15]. LDH consists of A and B subunits that assemble into homotetramers or heterotetramers forming 3 types and are distributed in the body in combinations. The first is LDHA that is most active in skeletal muscle, whereas the second is LDHB that is abundantly expressed in cardiac muscle. The third member of the family is LDH-C gene. It is expressed only in male [17, 39] and female [8] germ cells, and its protein product forms the enzymatically active homotetramer, classically referred to as LDH-C4. It is abundant in spermatocytes, spermatids, and sperm but found in modest amount in oocytes. LDH-C4 is required for spermatozoa motility, capacitation and penetration of zona pellucida [27]. In our experiment, LDH-C4 was down-regulated in TMX-treated group so the motility of this group was significantly decreased.

FSH-B, LH-B and GnRHR were synthesized and secreted from pituitary gland under effect of GNRH that is released from hypothalamus [28].nAChR, target for neonicotinoids is essential for controlling hypothalamic pituitary axis. Therefore, they are abundant and expressed in high level in the hypothalamus and any disturbance in this axis disturbs the secretion of neuropeptides from

hypothalamus [5].Neonicotinoids were revealed to pass blood brain barrier with unknown mechanism. They and their metabolites were found in the brain[33].Therefore, overstimulation of hypothalamic nAChRs was evidenced that resulted in influx of calcium ions inside the cells. The overflow of calcium ions inside cells results in activation of inducible nitric oxidase synthase (iNOS) because iNOS exhibits activation mode dependent on calmodulin binding to intracellular calcium ions[23, 24]. Excessive stimulation of iNOS results in excessive release of NO and NO peroxynitrate, that contributes greatly to oxidative stress, as demonstrated by Duzguner and Erdogan [13]. Consequently, oxidative stress interferes with the hypothalamic pituitary axis and causes neurochemical alteration. Moreover, it leads to increase level of apoptosis and neuronal degeneration. In the same context, imidacloprid was resulted in inactivation of acetylcholinesterase and subsequent accumulation of acetylcholine in synaptic cleft resulting in overstimulation of nAChRs [21]. Also, Abd-Elhakim et al. revealed that imidacloprid increased apoptotic index and oxidative stress in brain of adult and adolescent rat and affected negatively neurobehavioral performance[1]. As demonstrated above, down regulation of GnRHR, FSH and LH genes was expected as evidenced in our results. In conclusion, our research suggested that exposure to TMX for prolonged period has a adverse effects on the reproductive hormones and their expression. Therefore–,TMX may poses reproductive risk potential on mammalian reproduction.

Compliance with ethical standards

Experiment was performed under the guidelines for care and use of laboratory animals of the National institutes of Health (NIH), Egypt.

COMPETING INTERESTS DISCLAIMER:

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Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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