Deterioration of Cytochrome C Content and Mitochondrial Dysfunction in Brain of Male Rats after Sub-Chronic Exposure to Thiamethoxam and Protective Role of N- Acetylcysteine

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ABSTRACT

Mitochondria sustain healthy brain function. Herein we aimed to evaluate the thiamethoxam (MX) effect on the rat brain mitochondria in addition to the protective role of N-acetylcysteine (NAC) against MX harmful effects. Thiamethoxam was administered orally with five doses each week for 28 days to male albino rats at 1/50 of the LD_{50} (31.26 mg/kg bw). The results demonstrated that the thiamethoxam neurotoxicity was confirmed by the significant rising in acetylcholinesterase, and lactate dehydrogenase activities of plasma. A significant increase in mitochondrial antioxidants as superoxide dismutase and reduced glutathione was found. Also, significant induction of the oxidative stress and DNA damage via rising the malondialdehyde, and 8-hydroxy-2'-deoxyguanosine biomarkers was recorded by 32.5% and 118.61% respectively. Substantial depression in mitochondrial NADH dehydrogenase, cytochrome c reductase, cytochrome c oxidase, and Mg^{2+} ATPase complexes as well as 23 % cerebral infarction was manifested by histological evaluation using the dehydrogenase activity indicator, 2, 3, 5-triphenyl tetrazolium chloride staining. In conclusion, MX can pose a hazard to the integrity and functioning of rats' brain mitochondria, perhaps leading to neurodegenerative disorders. Additionally, earlier treatment of the synthetic antioxidant N-acetylcysteine could prove beneficial in combating the harmful effects of thiamethoxam.

Key words*:* Brain, NADH dehydrogenase, Cytochrome c, DNA damage, Neonicotinoid.

INTRODUCTION

Neonicotinoids (Ns) in agrochemical business, accounting for around 24% and 80% of global agrochemical and seed treatment sales, respectively (Maloney *et al*., 2017). The hydrophobicity of neonicotinoids, which is stronger than nicotine, has been related to their penetration into the mammalian central nervous system (Yamamoto *et al*., 1998). Thiamethoxam (MX) is a neonicotinoid pesticide that belongs to N-nitroguanidine class, and classified as a neurotoxin that influences mammalian cholinergic functioning via the stimulation of neuronal nicotinic acetylcholine receptors (nAChR) (Butcherine *et al*., 2019; Houchat *et al*., 2020). In acute oral and dermal investigations, it has low acute toxicity to rats (LD_{50} 1530 mg kg^{-1}) and is indexed as toxicity category III, with NOAEL = 61 mg/kg bw/day; LOAEL = 158 mg/kg bw/day in a chronic mammalian reproductive study (EPA, 2020).

MX is used to manage pests in a variety of crops, including sorghum, barley, canola, corn wheat, and cotton, at various stages of development (Schaafsma *et al*., 2016; Paquet-Walsh *et al*., 2019). Almost all of these crops are consumed as whole grains or used as meals in chicken and animal feed, posing a toxicity risk in the form of residues and metabolites (Butcherine *et al*., 2019). Although the mechanism of neonicotinoid on insect-nAChRs is well understood, few studies have been conducted to determine the *in vivo* effects of this pesticide family on mammals (Tomizawa *et al*., 2005). MX can have negative effects on rats' cholinergic system, causing behavioral and biochemical changes (Rodrigues *et al*., 2010). In addition, (Khaldoun-Oularbi *et al.*,2017) found that oral administration of MX altered some biochemical markers correlated with histopathological symptoms in the kidney, liver, and brain of exposed rats.

Pesticides attack mitochondria as one of their primary targets. Mitochondria are the site to the tricarboxylic acid cycle (TCA)-cycle and oxidative phosphorylation (OXPHOS) that produce the adenosine triphosphate (ATP) (Hatefi, 1985). Dysfunctions of this basic process have been linked to a variety of disorders and the onset of old age (Niccoli *et al*., 2012). Even if pesticides were only intended to harm plants, fungi, or insects, there is now growing evidence that they can impair mammals' mitochondria and create abnormalities in energy metabolism, which can lead to disease and neurotoxicity (Farkhondeh *et al*., 2020; Ko *et al*., 2020).

Several investigators documented the influence of neonicotinoids and other insecticides in mammalian bioenergetics systems. Imidacloprid interacts exclusively with F_oF_1-ATP synthase, resulting in functional inhibition of the enzyme and impaired the mitochondrial bioenergetics (Bizerra *et al*., 2018). In

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addition, chlorpyrifos, cypermethrin, and imidacloprid induced hepatomitochondrial dysfunction in male albino rats as a result of the treatments by the 1/50 of the LD_{50} of each insecticide (Taha *et al.*, 2021).

8-hydroxy-2′-deoxyguanosine (8-OH-2DG) is the most common biomarker of DNA damage that is frequently used for evaluating cellular oxidative stress. 8-OH-2DG has been linked to aging and the onset of neurological diseases like Alzheimer's (AD)(Cheng *et al.*, 1992). The increased mitochondrial 8-OH-2DG levels have been linked with increased mutation, fragmentation, deletion, and loss of mtDNA (Wei *et al.*, 2009; Qian *et al.*, 2010). Mitochondrial DNA damage can activate a chain of events such as reduced transcription and partial assembly of the electron transport chain (ETC) complexes, which leads to decreased ETC activity, thus lowering ATP synthesis, increased reactive oxygen species (ROS) production, and apoptosis (Gredilla *et al.*, 2010; Chan, 2012).

N-acetylcysteine is a low-cost aminothiol that works as a precursor to intracellular cysteine and reduced glutathione (GSH). As a result, it is regarded as a significant antioxidant since it enhances GSH biosynthesis, sustains intracellular GSH levels, and scavenges ROS (Sun, 2010). NAC is found to protect rats by lowering the rate of glyphosate-mediated apoptosis and oxidative stress in testis (Bhardwaj *et al.*, 2021), liver, kidney, and brain tissues (Turkmen *et al.*, 2019). Many pesticides' toxicity is mitigated by NAC such as organophosphate compounds (Osman *et al.*, 2021), alpha-cypermethrin (Arafa *et al.*, 2015), carbamates (Dhouib *et al.*, 2015), and imidacloprid (Hussein *et al.*, 2018). Several studies have shown that NAC improves mitochondrial function and reduces behavioral deficits in the R6/1 mouse model of Huntington's disease (Wright *et al.*, 2015), enhances mitochondrial reduced glutathione (mtGSH) content and mitochondrial function in oligodendroglia (Zhou *et al.*, 2021), and protects mitochondria from oxidative injury (Kuo *et al.*, 2009).

This study was aimed to estimate the mitochondrial dysfunction and oxidative stress induction in the brain of male albino rats exposed to subchronic thiamethoxam intoxication. So, plasma lactate dehydrogenase (LDH) and acetylcholinesterase (AChE), mitochondrial respiratory chain complexes I, III, IV, and V (Mg^{+2} ATPase) were assessed. Oxidative stress (MDA, and 8-OH-2DG) and antioxidant biomarkers (SOD and GSH) were measured. In addition, the histological analysis of the extent of brain damage is estimated with the mitochondrial activity indicator. In addition, the protective effect of NAC against MX-induced neurotoxicity in male rats was evaluated.

MATERIAL AND METHODS

Insecticide and chemicals

Thiamethoxam (MX) (98%) was supplied from Zhejiang Heben pesticide and chemical Co., Ltd. China. N-acetylcysteine; NAC (600 mg as effervescent instant) was provided by Sedico Pharmaceuticals Company in Egypt. Wholly other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Animals

Mature male albino rats, weighing 144 ± 3 g, were supplied by the animal house, Faculty of Science, Alexandria University. They were arbitrarily housed in a plastic cage, and observed under laboratory environments; 25 ± 1 °C, 65–75 % humidity, and a normal light/dark cycle. Animals were fed on a well-balanced rodent diet (60 % maize, 20 % soybean, 0.2 % vitamins, 1.5 % brown dust, 0.5 % salt, and 3 % mollus) that was obtained from Animals Food Manufactory of the Agriculture Ministry, Embaba, Giza, Egypt, and water *ad libitum* for two weeks before being tested. Handling of animals was achieved according to the guidelines for care and use of laboratory animals and confirmed by (OECD, 2008).

Experimental design

Twenty male albino rats were randomly divided into four groups, 5 animals per each, as follows: the first was kept as a control group, where animals orally received corn oil (0.5 ml /kg bw). In the second group (NAC), freshly prepared N-acetylcysteine was intubated for the rats at a dose of 150 mg/kg bw. The rats of the intoxicated group (MX) were administrated 31.26 mg/kg bw of thiamethoxam. The last group was served as a co-treated (NAC + MX), where the animals received 150 mg/kg bw of N-acetylcysteine 30 min before thiamethoxam (31.26 mg/kg bw) administration. The selected dose of MX was equivalent to 1/50 of the acute oral median lethal dose (LD_{50} = 1563 mg/kg body weight) according to (Tomlin, 2009). All rats have received the corresponding doses five times a week by gastric intubation for one month.

Sample preparation

By the end of the experimental period, animals abstained from food overnight with free access to water, and were euthanized using light anesthesia of sodium pentobarbital. The blood was withdrawn from retro-orbital plexus in EDTA coated tubes. The plasma samples were separated at 1500 xg for 10 min using Sigma K30 bench centrifuge and kept at -20 °C for biochemical analysis. In addition, the brain from each animal was quickly removed, washed with ice-cold normal saline, and frozen until assessing.

Isolation of rat brain mitochondria and mitochondrial DNA

Brain mitochondria were isolated using a modified protocol of Krause *et al.* (2005). Briefly, brain tissue was homogenized using a glass Teflon homogenizer with 5 volumes (w/v) of ice-cold buffer containing 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH, 320 mM sucrose, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4, and centrifuged at 1300 xg for 3 min at 4 °C. Subsequently, the obtained pellet was extracted twice with 2 and 1 ml homogenization buffer, respectively. The combined supernatants were re-centrifuged at 17,000 xg for 10 min at 4 °C. Finally, the pellet was washed and re-suspended in 0.5 mM PMSF and 320 mM sucrose. Isolated mitochondria were stored in aliquots at -20 °C. Mitochondrial DNA (mtDNA) was extracted using a kit from BioVision research products (Mountain View, CA, USA).

Determination of protein

The protein content of plasma and mitochondrial preparations was assayed by the method of Lowry *"et al.*, (1951)" using bovine serum albumin as a standard using T-80+UV/VIS spectrometer PG Instruments Ltd. (Leicestershire, UK).

Biochemical Assessment

1. Acetylcholinesterase (AChE) activity

The activity of AChE in plasma was measured at 412 nm using acetylthiocholine iodide (ATChI) as a substrate, as described by (Ellman *et al.*, *1961*). One unit of AChE activity was expressed as nmole hydrolyzed ATChI/min/ml.

2. Lactate* dehydrogenase *(LDH) activity

The activity of LDH in plasma was determined by the method of Oba and Uriteni (1982).

The activity of LDH is indicated by the decrease of absorbance at 340 nm for 3 min, which is related to the amount of NADH oxidized according to the following reaction:

$$\text{Puruvate} + \text{NADH} + \text{H} \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

One unit of LDH activity was expressed as µmole oxidized NADH/min/ /ml.

3. Mitochondrial NADH dehydrogenase (Complex I) activity

NADH dehydrogenase activity was determined using Galante *et al.* (1978). The procedure depends on the oxidation of NADH by incubation of mitochondrial protein (40 µg /ml) in the mixture containing 40 mM phosphate buffer, pH 7.4, 0.1% sodium cholate, 1.5 mM potassium cyanide, and 1.3 mM potassium ferricyanide, as an electron acceptor for 1 min at 30 °C. The reaction was started by adding 0.14 mM NADH, and the change in absorbance at 340 nm was monitored for 3 min. The results were reported as nmol NADH oxidized/min/mg protein.

4. Mitochondrial cytochrome c reductase (Complex III) activity

The reduction of mitochondrial cytochrome c reductase was measured calorimetrically at 550 nm according to Green "*et al.*, (1963)". Mitochondrial enzyme 10 µl was added to 1 ml of the assay system, which contained 50 mM phosphate buffer, pH 7.4, 0.1 mM EDTA, 1 mg/ml cytochrome c, 1.6 mM potassium cyanide, 1 mM NADH, and 10% sodium cholate. The increase in optical density was taken every 15 sec for 2 min at 38 °C. The specific activity was expressed as µmole cytochrome c reduced /min/mg protein. The extinction coefficient of reduced cytochrome c was 18.5×10^6 cm^2/mole.

5. Mitochondrial cytochrome c oxidase (Complex IV) activity

The oxidation of cytochrome c was spectrophotometrically measured by Green "*et al.*, (1963)". Briefly, in a 1 ml of the assay system containing 50 mM phosphate buffer, pH 7.4, 0.1 mM EDTA, 1 mg/ml cytochrome c, and 10% sodium cholate, 20 µl of the mitochondrial enzyme was added. The decrease in absorbance was recorded every 15 sec for 2 min at 550 nm. The enzyme activity was expressed as mmole cytochrome c oxidized/min/mg protein. The extinction coefficient of oxidized cytochrome c was 19.6 mM^{-1} $cm^{-1.}$

6. Mitochondrial Mg^{2+} ATPase (Complex V) activity

The assay relies on the determination of the amount of inorganic phosphate created from the hydrolytic reaction of ATP by the ATPase. Mitochondrial protein (1 mg/ml) was added to a medium containing 20 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$ and 5 mM ATP. The mixture was incubated for 5 min at 37 °C in a shaking water bath. The reaction was stopped by the addition of 5% trichloroacetic acid (TCA), and then inorganic phosphate (Pi) was determined at 740 nm (Taussky *et al.*, 1953). The activity of ATPase was expressed as µmole Pi/min/mg protein.

7. Superoxide Dismutase (SOD) activity

SOD as an enzymatic antioxidant was estimated spectrophotometrically at 25 °C by the method of Marklund and Marklund (1974) with some modifications. The assay medium was contained 50 mM Tris–HCl, pH 8.0, and 0.24 mM pyrogallol in a

total volume of 1.0 ml. The autoxidation of pyrogallol was monitored at 420 nm for 3 min in the absence and presence of the enzyme. At least three concentrations of the enzyme that produced between 30 to 60 % inhibition of pyrogallol autoxidation were used. One unit of enzyme (U) activity is defined as the amount that produced 50% inhibition of pyrogallol autoxidation under the standard assay conditions. Mitochondrial SOD activities were expressed as µmol oxidized pyrogallol/mg protein/ml.

8. Reduced Glutathione (GSH) content

Reduced GSH is a non-enzymatic antioxidant was measured according to Ellman (1959). The mitochondrial suspension in 0.1 M phosphate buffer, pH 7.4 was added to an equal volume of 5 % TCA. The mixture was allowed to stand for 30 min at RT before centrifugation at 3400 xg for 15 min. The assay was performed with 0.5 ml of supernatant, 1 ml of 0.1 M phosphate buffer, pH 8.0, and 0.02 ml of Ellman's reagent (39.6 mg of 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB), 15 mg $NaHCO_3$ in 10 ml 0.1 M phosphate buffer, pH 7.0). After incubation for 3 min, the absorbance was recorded at 412 nm. The amount of reduced glutathione was calculated from the GSH standard curve as µmole glutathione/mg protein.

9. Lipid peroxidation (LPO) level

The lipid peroxidation process is determined by the thiobarbituric acid (TBA) method which estimates the malondialdehyde (MDA) formation according to Buege *"et al,.* (1978)". Briefly, 0.5 ml of mitochondrial suspension was mixed well with 2 ml of TBA reagent, which contains 0.375 % TBA, 15 % TCA, and 0.25 N HCL. The mixture was incubated for 15 min in a boiling water bath. After cooling, the mixture was centrifuged at 3000 xg for 20 min. The MDA level was measured spectrophotometrically at 535 nm. The amount of thiobarbituric acid reactive substance (TBARS) in mitochondrial suspension was calculated using the molar extinction $\mathcal{E}$ = 155 $mM^{-1}cm^{-1}$ and the results were expressed as nmol MDA/mg protein.

10. Assessment of mitochondrial DNA damage

8-OH-2DG content in brain mitochondria was measured by Sandwich-ELISA (enzyme-linked immunosorbent assay) method. Analyzes were performed using Quick Detect™ 8-OHdG (Rat) ELISA Kit, BioVision, USA (Catalog # E4442-100). Absorbance at 450 nm was recorded by a microplate reader (STAT FAX-2100, Awareness Technology, Inc., Palm City, Florida, USA). The 8-OH-2DG content is expressed as ng 8-OH-2DG /mg protein.

Histological evaluation

Cerebral infarction area was detected by the 2, 3, 5-triphenyl tetrazolium chloride staining according to Bederson *"et al.,* (1986)". Individual slices were consecutively and equidistantly taken from the front to the back, spacing 2 mm. The slices were stained with stirring in a solution of 1% TTC in 0.2 M phosphate buffer, pH 7.4 at 37˚C for 20 min and placed in 4% phosphate-buffered formalin for preservation. Normal brain tissue was stained red and infarct tissue white. Tracings of infarcted areas were made from the photographs and quantified by an imaging analysis system (Image J software program). Total brain and infarct areas were automatically determined based on total pixel intensity and area after segmentation of the red and green images, respectively. The statistical significance of differences in infarct size was expressed as a percentage of infarcted area to the total brain area.

Statistical analysis

Data have been expressed as mean ± standard error (SE). The statistical significance criterion was set at $p \leq 0.01$. For data analysis, one-way variance analysis (ANOVA) was employed, followed by the Student-Newman-Keuls Test uses the IBM SPSS software version 25.0 (Statistical Package for Social Sciences, Chicago, IL, USA) (IBM Corp, 2017).

RESULTS AND DISCUSSION

RESULTS

Changes in plasma enzyme activity can be used to detect and pinpoint tissue cell damage or proliferation, as well as to track the disease's therapy and progression. Treatment with thiamethoxam (MX) significantly ($p \leq 0.01$) increased the activity of AChE in plasma by 68.8 % compared to control animals (Table 1). In addition, N-acetylcysteine (NAC) treatment in the absence of MX showed a significant change in the enzyme activity by 2.24 %. The present study indicated that pre-supplementation with NAC to the rats intoxicated with MX alleviated its negative effect by 23.76 % on the AChE activity.

LDH activity is normally recognized as a diagnostic marker of tissue damage. Table 1 shows the effect of MX and NAC treatment on plasma LDH activity in male rats. The results indicate a significant ($p \leq 0.01$) elevation of plasma LDH activity in MX treated rats by 563.6 % when compared to control rats. NAC treatment alone did not exert any significant ($p \leq 0.01$) effect on LDH activity. However, pretreatment of MX exposed rats with NAC resulted in significant recovery ($p \leq 0.01$) of LDH activity to be 148.67 %.

Measurement of the individual enzymes associated with mitochondrial oxidative phosphorylation (OXPHOS) forms a key part of the diagnostic of mitochondrial disorders. Table 2 reveals the negative influence of MX on mitochondrial bioenergetics and cytochromes c contents in exposed rat brains. Data indicated that the MX dosing significantly ($p \leq 0.01$) decreased complex I (NADH dehydrogenase) activity by 44.52 %, while supplementation with NAC modified the MX toxic effect to 26.47 % relative to the control group. The complex III (cytochrome c reduced) activity of brain mitochondria was significantly ($p \leq 0.01$) decreased by 17.35 % in rats given MX. However, concomitantly supplementation by NAC attenuated the MX effect to be 10.20 % compared to control. In addition, the complex IV (cytochrome c oxidized) activity was significantly ($p \leq 0.01$) inhibited by 44.32 % due to MX treatment, however, pre supplementation with NAC noticeably alleviated the MX harm effect to 16.6 % paralleled to control (Table 2). Likewise, a significant decrease in the mitochondrial complexV (Mg^{2+} ATPase) activity by 27.02 % for rat brains was observed after MX administration. While the MX administration after NAC was enhanced the MX effect to 13.16 % compared to control.

The effect of 1/50 LD_{50} exposure to MX for a month on the oxidative stress parameters in the brain mitochondria of male rats is illustrated in Table 3. Treatment with NAC alone was caused a significant ($p \leq 0.01$) decrease in SOD activities by 6.5 %, and an increase in the levels of GSH by 22.36 %, while the MDA concentrations were non-significant different from the control. The results revealed that, SOD, GSH and MDA levels were significantly ($p \leq 0.01$) increased by 11.8 %, 82.9 %, and 52.4 %, respectively in rats treated with MX. In addition, the presence of NAC with MX was exhibited a significant ($p \leq 0.01$) reduction in the elevation of SOD, GSH, and MDA by 14.2 %, 39.6 %, and 32.5 %, respectively compared to the control group.

The 8-OH-2DG content in brain mitochondria of male rats was shown as a DNA damage biomarker and presented in Fig. 1. The 8-OH-2DG level significantly increased by 1.95 % in the NAC-treated group compared to the control. It was found that rats exposed to MX showed significant ($p \leq 0.01$) elevation in 8-OH-2DG levels by 118.61 %. while the animal group supplied NAC with MX treatment greatly ($p \leq 0.01$) attenuated the DNA damaging effect of MX to be 40.32%.

TTC staining was used to determine the extent of cerebral infarction. Normal brain tissue was stained red, while infarct tissue was stained white as shown in Fig 2 and Table 4. Brain section of rats intoxicated with MX showed a significant ($p \leq 0.01$) histological damage represented by 23% infarct tissue, mainly in the frontal, apical, and temporal cortex (Fig. 2). NAC supplementation significantly ($p \leq 0.01$) attenuated the cerebral infarcted area to be 6 %.

Table 1. Effects of thiamethoxam (31.26 mg/kg bw), N-acetylcysteine (150 mg/kg bw), and their combination on acetylcholine esterase and lactate dehydrogenase of exposed rat plasma

Animal groups	AChE (nmole hydrolyzed ATChI/min/ml)	LDH (µmole oxidized NADH/min/ /ml)
Control	$0.446^{d} \pm 0.004$	$5.63^{c} \pm 0.033$
N-acetylcysteine	$0.456^{c} \pm 0.004$	$5.00^{c} \pm 0.057$
Thiamethoxam	$0.753^{a} \pm 0.0024$	$37.36^{a} \pm 0.69$
N-acetylcysteine + thiamethoxam	$0.552^{b} \pm 0.001$	$14.00^{b} \pm 0.063$

Values denote the mean ± SE of five animals/group. Superscripts different letters indicating statistical significant differences between groups at $p \leq 0.01$ using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Test.

Table 2. Effects of thiamethoxam (31.26 mg/kg bw), N-acetylcysteine (150 mg/kg bw), and their combination on the mitochondrial bioenergetics and cytochromes c contents in exposed rat brain

Animal groups	Complex I (nmole NADH oxidized /min/mg protein)	Complex III (µmole cytochrome c reduced/min/mg protein)	Complex IV (mmole cytochrome c oxidized/min/mg protein)	Complex V (µmole Pi/min/mg protein)
Control	$197.66^{a} \pm 0.33$	$0.196^{a} \pm 0.001$	$61.30^{a} \pm 0.75$	$85.13^{a} \pm 0.12$
N- acetylcysteine	$189^{b} \pm 0.57$	$0.188^{b} \pm 0.001$	$53.73^{b} \pm 0.57$	$86.01^{a} \pm 0.48$
Thiamethoxam	$109.66^{d} \pm 0.86$	$0.162^{d} \pm 0.003$	$34.13^{d} \pm 0.12$	$62.13^{c} \pm 0.23$
N-acetylcysteine + Thiamethoxam	$145.33^{c} \pm 0.34$	$0.176^{c} \pm 0.005$	$51.13^{c} \pm 0.20$	$73.93^{b} \pm 0.03$

Values denote the mean ± SE of five animals/group. Superscripts different letters indicating statistical significant differences between groups at $p \leq 0.01$ using one-way analysis of variance (ANOVA) followed by the Student-Newman- Keuls Test.

Table 3. The oxidative stress status in brain mitochondria of rats treated with thiamethoxam (31.26 mg/kg bw), N-acetylcysteine (150 mg/kg bw), and their combination for a month (5 doses/week)

Animal groups	SOD (µmol oxidized pyrogallol/mg protein/ml)	GSH (µmole/mg protein)	MDA (nmole/mg protein)
Control	$24.6^{b} \pm 0.049$	$10.51^{d} \pm 0.020$	$0.126^{c} \pm 0.0002$
N-acetylcysteine	$23.0^{c} \pm 0.041$	$12.86^{c} \pm 0.041$	$0.124^{c} \pm 0.0005$
Thiamethoxam	$27.5^{a} \pm 0.17$	$19.22^{a} \pm 0.052$	$0.192^{a} \pm 0.0024$
N-acetylcysteine + Thiamethoxam	$21.1^{d} \pm 0.45$	$14.67^{b} \pm 0.014$	$0.167^{b} \pm 0.0006$

Values denote the mean ± SE of five animals/group. Superscripts different letters indicating statistical significant differences between groups at $p \leq 0.01$ using one-way analysis of variance (ANOVA) followed by the Student-Newman- Keuls Test.

Table 4. Determination of infarcted area by TTC as a percentage of brain section of rats treated with thiamethoxam (31.26 mg/kg bw), N-acetylcysteine (150 mg/kg bw), and their combination for a month

Animal groups	Infarcted area (%)
Control	$1.0^{c} \pm 0.05$
N- acetylcysteine	$1.5^{c} \pm 0.06$
Thiamethoxam	$23^{a} \pm 0.54$
N- acetylcysteine + Thiamethoxam	$6.0^{b} \pm 0.09$

Values denote the mean ± SE of five animals/group. Superscripts different letters indicating statistical significant differences between groups at $p \leq 0.01$ using one-way analysis of variance (ANOVA) followed by the Student-Newman- Keuls Test.

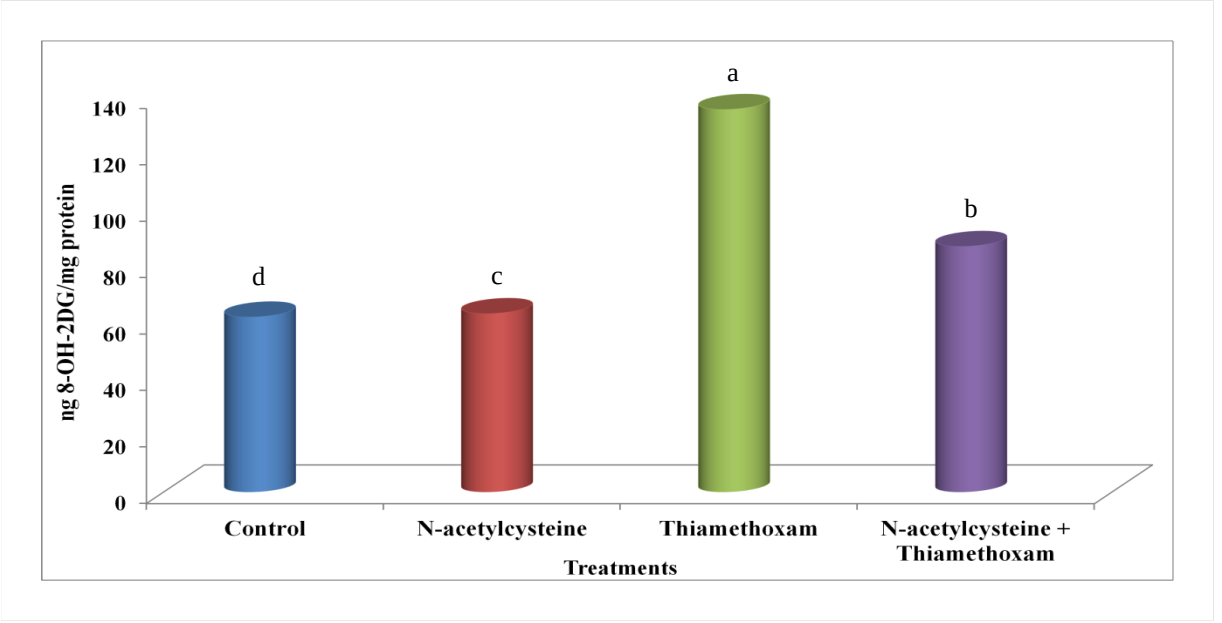

Fig.1. Assessment of mitochondrial DNA damage in the brain of rats treated with thiamethoxam (31.26 mg/kg bw), N-acetylcysteine (150 mg/kg bw), and their combination for a month. Values denote the mean ± SE of five animals/group. Superscripts different letters indicating statistical significant differences between groups at $p \leq 0.01$

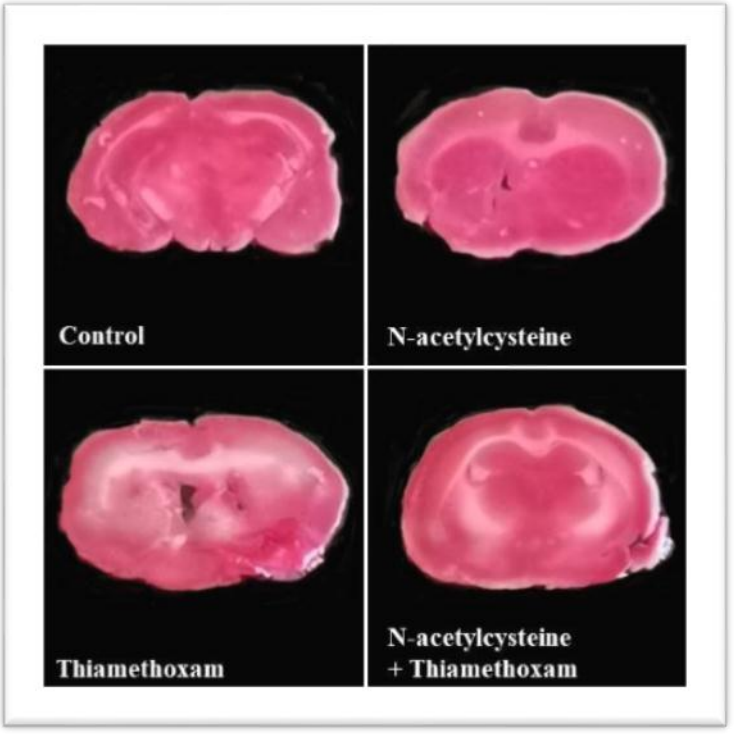

Fig.2. Rat brain sections stained by TTC after treatment with a sublethal dose of thiamethoxam (31.26 mg/kg bw), N-acetylcysteine (150 mg/kg bw), and their combination for a month (5 doses/week)

DISCUSSION

Neurotransmission is affected by neonicotinoid insecticides; hence the cholinergic neurotransmitter system is likely to be the most harmed in mammals. This system is important for the control of cognitive functions, and its dysfunction is linked to the onset of a multitude of neurodegenerative disorders (Hampel *et al.,* 2018). Current data revealed a marked increase in plasma acetylcholinesterase (AChE) as a result of MX treatment. In agreement with Abou-Donia *et al.* (2008) who observed the possibility of neurotoxicity after in utero imidacloprid exposure. During behavioral assessments on postnatal day 30, the offspring of treated moms showed severe sensorimotor deficits. deficits, increase the AChE activity (125–145 %) in the midbrain, cortex, and brainstem, and (125 %) in plasma. Rodrigues *et al.* (2010) mentioned that thiamethoxam may affect the nAChRs in the central nervous system of rats, this might change the cholinergic transmission, affecting anxiety, and altering AChE activity. In contrast, Kapoor *et al.* (2014) claimed that both acute and chronic treatment of imidacloprid in the rodent resulted in significant suppression of brain AChE activity, despite no significant effects being reported in other research (Katić *et al.,* 2021). A study by Akbel *et al.* (2018) showed that malathion inhibited the AChE activity in rat serum. In addition, Hussein (2018b) revealed a decrease in plasma cholinesterase levels of rats exposed to deltamethrin (6 mg/kg/ day) for a week. Herein the current results revealed that thiamethoxam may harm the mammal's nervous system as evidenced by the rise in plasma AChE activity.

In the human body, LDH is involved in the anaerobic conversion of pyruvate to lactate and conversely. Lactate levels are widely used in clinical treatment as a marker of sickness severity and response to medical therapies. The current work showed an increase in plasma LDH activity of the MX intoxicated rats. The predominance of anaerobic conditions induced by MX toxicity is demonstrated by the elevation of LDH. This suggested that the metabolic pathway may have become anaerobic to achieve the higher energy demands during MX's toxic stress. Similar to our findings, Feki *et al.* (2019) posted that MX elevates LDH activity in the plasma of treated female rats. In rats orally treated by MX (100 mg/kg bw for seven days), El Okle *et al.* (2016) found a significant rise in the activity of serum LDH. Additionally, LDH activity underexposure of imidacloprid was significantly increased in the rats' brains (Lonare *et al.,* 2016). Also, Mongi *et al.* (2021) observed that serum LDH activity was increased in chlorpyrifos intoxicated rats. Glyphosate, fipronil, microcystin-LR, and other toxicants have been shown to increase LDH activity in numerous mammalian organs Ahmad *et al.* (2017).

Mitochondria are the primary energy source, supplying NADH and ATP via oxidative phosphorylation to maintain neuronal homeostasis and function. So, it's not surprising that mitochondrial dysfunction is a major mechanism of drug-induced toxicity, as well as the primary mechanism in several pathologies and a major toxicological target (Bergman *et al.,* 2016). MX intoxication impaired the rat brain mitochondria, as evidenced by the reduction in mitochondrial NADH dehydrogenase. This observation could be explained by the enzyme complex's configurational structure, which is made up of at least 40 polypeptides tightly attached in the inner mitochondrial membrane (Küffner *et al.,* 1998). The lack of NADH oxidation lowers NAD+ levels significantly, preventing the activation of several NAD+-dependent dehydrogenases in the β-oxidation and the tricarboxylic acid (TCA) cycle pathways (Massart *et al.,* 2013). In consistent the with present data, Agrawal *et al.* (2015) discovered that cypermethrin changes the mitochondrial proteome, lowers membrane potential, reduces mitochondrial complex I activity, and enhances apoptosis in rat neurons. The negative consequences of the mitochondrial NADH dehydrogenase inhibition revealed by the present study illustrated that impairment of the TCA cycle can result in hyperlactatemia and lactic acidosis because the conversion of metabolic pyruvate to lactate by lactate dehydrogenase is promoted by NADH buildup (Margolis *et al.,* 2014).

Within mitochondria, Complex III is a major source of reactive oxygen species (ROS). In ischemia-damaged mitochondria, a malfunction of the iron-sulfur protein's catalytic domain prevents electron transport to cytochrome c_1, lowering complex III activity (Lesnefsky *et al.,* 2001). The findings demonstrated a considerable decrease in the activity of brain mitochondria complex III. Agreeing with Karami-Mohajeri *et al.* (2013), mevinphos acute exposure resulted depletion in the NADH cytochrome C reductase and succinate cytochrome C reductase (Complexes II and III) in pheochromocytoma cell line. Unlike, Lee e*t al.* (2021) proposed that two chlorinated hydrocarbons, chlordane and β- hexachlorocyclohexane, were linked to increased mitochondrial NADH dehydrogenase (complex I) and cytochrome c reductase (complex III) specific activity in treated zebrafish embryos.

Complex IV is the respiratory chain's final electron acceptor that catalyzes the reduction of O_2 to H_2O (Castresana *et al.,* 1994). Our findings revealed a significant reduction in complex IV activity in brain mitochondria, which is consistent with previous

research in acutely monocrotophos- and dichlorvos-treated rats, generated organophosphate-induced delayed neuropathy that evidenced by the suppression of complexes I, II, and IV activity in the cortex, cerebellum, and brain stem (Masoud *et al.,* 2009). In addition, Delgado *et al.* (2006) found that malathion produces oxidative stress in the central nervous system (CNS) of intoxicated rats, possibly due to the inactivation of mitochondrial respiratory complexes (II and IV). Furthermore, Wani *et al.* (2011) cited that increased cytosolic cytochrome c levels and caspase activation in OP-exposed animal models suggest a role for mitochondria deficit-induced apoptosis in neuronal death. Also, Abdel-Mobdy *et al.* (2019) reported that technical and formulated dimethoate promoted respiratory system activities (cytochrome c oxidase and succinate cytochrome reductase) in male albino rats' brain, liver, and kidney tissues.

ATP synthase (complex V) is a reversible enzyme that can hydrolyze ATP as well as synthesize it from ADP and inorganic phosphate (Pi). Mühleip *et al.* (2019) describe it as a multicomponent structure that crosses the inner membrane of mitochondria, the cell's energy providers. According to this study, MX intoxication inhibits Mg^{2+} ATPase function in the rat brain. It can deduce that thiamethoxam poisoning reduced complex V activity by disturbing calcium homeostasis and increasing mitochondrial membrane permeability. In agreement with Abdel-Razik and Hamed (2015)'s study, abamectin has a direct effect on F_oF_1-ATPase (complex V) and functions similarly to oligomycin, a complex V specific inhibitor. Inline, Imidacloprid-treated mice have reduced brain mitochondrial ATPase activity, (Abdel-Razik, 2019). Also, intoxication with lambda-cyhalothrin suppressed complex I and V activities in rats' liver (Abdel-Razik, 2017). In contrast (Delgado-Coello *et al*., 2006) proposed that increased cytoplasmic calcium activates several enzymes that cause membrane damage, and ATPases being one of the most important enzymes implicated.

Antioxidant enzymes represent primary and secondary defensive actions that prevent or neutralize reactive oxygen species (ROS) and their metabolites into harmless stable molecules (Rahman, 2007). SOD, a common enzymatic antioxidant, protects the cells from free radical-induced oxidative stress by catalyzing the conversion of superoxide anion free radicals to O_2, which is then transformed by glutathione or catalase (CAT) to H_2O_2 (Thowfeik, 2016). In the current investigation, the MX-treated group demonstrated a significant rise in SOD and GSH activities, as well as in MDA levels. In agreement with these findings, several researchers, including Ahmadi *et al.* (2018), found a 61.0 percent increase in SOD, which they attributed to pesticide-induced overproduction of reactive species (Hernández *et al.,* 2013). El-Gendy *et al.* (2010) found a dramatic increase in SOD activity of male mice exposed to imidacloprid. Vidyasagar *et al.* (2004) posted that patients with organophosphorus pesticide exposure had higher RBC and plasma SOD levels than the control group. Yan *et al.* (2016) Also found that MX caused oxidative stress in zebrafish livers, as seen by increased SOD, CAT, and GPx activity. Moreover, Taha *et al.* (2021) reported that chlorpyrifos, cypermethrin, and imidacloprid cause oxidative stress by forming free radicals, suggesting a dysfunction of the mitochondrial respiratory chain. In contrast, Akbel *et al.* (2018) recorded decreased SOD activity in brain tissues of malathion exposed rats. Studies in Egypt and Thailand have shown a decline in the SOD activity in the exposed workers (Wafa *et al.,* 2013; Sudjaroen, 2017). According to, Hernández *et al.* (2013) the decreased SOD activity may be a result of oxidative stress produced by pesticide exposure and subsequent superoxide radical accumulation. This result suggests that Mn-SOD is important for the redox status of mitochondria in cells and tissues (Buettner, 2011).

Mitochondrial GSH is a vital non-protein thio preventing excessive hydrogen peroxide buildup and subsequent oxidative stress (Marí *et al.,* 2013). The current research revealed a significant elevation of the GSH content in the rat brain mitochondria, which may be due to the increased requirement for GSH to counteract free radicals caused by MX. Consistent with this research, Shukla *et al.* (2017) reported that neonicotinoid imidacloprid caused a significant elevation in GSH levels in the brain, kidney, and liver of zebrafish. In contrast, other researchers reported significant reduction in serum and brain GSH content of rats exposed to malathion and deltamethrin, respectively (Akbel *et al.,* 2018; Hussein *et al.,* 2018). Also, Martín *et al.* (2000) reported a decreased GSH content in mitochondria causes H_2O_2 accumulation which can lead to lipid peroxidation and cell damage.

One known mechanism of mitochondrial failure in many disorders is the intensity of LPO (Negre-Salvayre *et al.,* 2010). The stimulation of LPO in mitochondrial membranes, combined with oxidative stress, permeability alteration, reduces membrane potential and detached oxidative phosphorylation (Batandier *et al*., 2004). Our data demonstrated a significant increase in MDA content due to thiamethoxam exposure, showing a loss of structural and functional integrity of the mitochondrial membrane. Mendes Arent *et al.* (2014) cited that MDA levels are one of the main pathways of secondary damage in traumatic brain injury. Pesticides have been known to trigger oxidative stress and lipid peroxidation in humans' and rats' brains (Verma *et al.,* 2003). In line with the current observed data Akbel *et*

al. (2018), revealed that malathion increased MDA content in the brain tissues of the exposed rat. In addition, Hussein *et al.* (2018) revealed that deltamethrin induced neurotoxicity in albino rats, as evidenced by a substantial increase in MDA levels in the blood and brain tissues.

8-OH-2DG is the most common single nucleotide-base lesion in nuclear and mitochondrial DNA that can cause mutations in replicating DNA. Pesticides bond covalently to DNA due to their extremely reactive nature and can impair metabolic processes by destroying genetic material (Jacobsen-Pereira *et al.*, 2018). Present data revealed a significant increase in the mitochondrial DNA 8-OH-2 DG of MX treated rat brain. Our result is consistent with the findings of Elhamalawy *et al.* (2022) who posted that MX significantly rises the DNA damage rates in hepatic and renal cells of treated mice as measured by diphenylamine test and comet assay. MX directly interacts with DNA, and has a significant impact on numerous biological and biochemical indices in treated animals Jameel *et al.* (2020). Furthermore, Hussain *et al.* (2020) found that MX increased DNA damage in hepatocytes, kidneys, and blood cells in fish, as determined by comet assay. Recently, Lovaković (2021) observed that giving rats low dosages of imidacloprid for 28 days induced DNA damage in leukocytes and brain cells. Also, El-Ela *et al.* (2019) *in vivo* investigated the potential genotoxicity of imidacloprid and discovered enhanced primary DNA damage in treated rats. In addition, Akbel *et al.* (2018) notice the increased 8-OH-2DG levels in brain tissues of malathion exposed rats.

The 2,3,5-triphenyl tetrazolium chloride is one of the histochemical staining procedures for infarcted tissue identification (Altman, 1976). The histological evaluation in this study revealed some necrotic changes in brain tissues of MX treated rats due to a lack of dehydrogenases activity, indicating irreversible brain ischemic damage, which is consistent with Kloner *et al.* (1981), and is supported by the above findings of MX toxic effect on brain mitochondria. Earlier investigators had diagnosed damaged tissues in various organs using TTC dye. Whereas, TTC has been used by Popp *et al.* (2009) to assess brain regions affected by focal ischemia in a rat model of stroke and also to evaluate early myocardial infarction in pig hearts perfused with blood (Khalil *et al.*, 2006).

N-acetylcysteine is a thiol-containing amino acid derivative that acts as a cysteine donor for the formation of GSH (Kawoos *et al.*, 2017). The current findings showed that NAC can protect the rats' brain mitochondria from oxidative stress and DNA damage caused by MX intoxication. This was achieved by modulating the activities of AChE, LDH, energetics, and antioxidant biomarkers; as complex I, III, IV, V, SOD, GSH content, and decreasing the MDA levels, as well as reducing DNA damage by lowering brain mitochondrial genotoxic lesion, 8-OH-2DG. In agreement with a study by *Peña-Llopis et al.* (2003) documented that NAC improves muscle GSH depletion and AChE activity in the European eels (Anguilla Anguilla) brain exposed to dichlorvos. Lasram *et al.* (2014) explain the improvement in AChE activity by the ability of NAC to accelerate the quick removal of toxic OP metabolites from the body and reduce their impact. In synaptic mitochondria, NAC was reported to boost mitochondrial complex I and IV specific activities both *in vitro* and *in vivo*, improve lipid composition, and restore ATPase activity to normal levels (Banaclocha, 2001; Kamboj *et al.*, 2006). Xiao *et al.* (2016) cited that NAC supplementation increased cell proliferation, antioxidation, and mitochondrial bioenergetics while decreasing cell apoptosis. These findings show that NAC may be able to reverse H_2O_2-induced intestinal damage. NAC therapy also improves the activity of complex I, II, III, and cytochrome c oxidase in rats, lowers the ADP/ATP ratio, elevates mitochondrial glutathione, and inhibits cytokine production, apoptosis, and nitrosative damage (Zalewska *et al.*, 2020). Inline, Bhardwaj *et al.* (2020) observed that NAC intake significantly reduced DNA damage caused by granulosa cell apoptosis in response to methoxychlor exposure, avoiding genotoxicity-induced cytotoxicity in granulosa cells. The current evidence is consistent with many prior findings stating that, NAC supplementation recovered the tested biochemical and genetic biomarkers in the chlorpyrifos-ethyl and chlorpyrifos-methyl exposed rat Osman *et al.* (2021). NAC's antioxidant and free radical sweeper activities have been shown to protect rats' blood, renal, hepatic, cardiac, and brain tissues from glyphosate-induced histopathological changes and oxidative stress (Turkmen *et al.* 2019). Also, Aboubakr *et al.* (2019) mentioned that NAC can be consumed as a preventive and therapeutic drug since it mitigates the biochemical, molecular, and histopathological deviations induced by malathion intoxication in rats livers. Similarly in rat brain tissue, NAC therapy exhibits antioxidant, anti-inflammatory, and anti-apoptotic properties against chlorpyrifos-induced neurotoxicity (Mahmoud *et al.*, 2019). According to Abouelella *et al.* (2018), NAC saves the reproductive system by reducing nicotine-induced oxidative stress and reproductive injury in male rats. Furthermore, Kheradmandi *et al.* (2019) found that injecting NAC intraperitoneally with chlorpyrifos reduces the pesticide's negative effects on mice testis. In agreeing with the previously mentioned reviewer the present finding demonstrated that NAC supplementation

showed a reduction in brain ischemic injury size suggesting its role in restoring dehydrogenase activity.

CONCLUSION

It can conclude be that, the 28 days of exposure to 31.26 mg/kg bw of thiamethoxam generated many toxic effects on the brain of rats. As manifested by the alteration in plasma AChE and LDH activities, impairment of mitochondrial respiratory chain complexes, change the enzymatic and non-enzymatic defense systems, induction of oxidative stress as revealed by the increase of both malondialdehyde (MDA) and DNA oxidation biomarker (8-OH- 2DG) levels as well as histological alterations. All the present aforementioned toxicological data revealed the abnormalities of the brain mitochondria. The study suggests that MX can pose a risk to the integrity and functioning of the rats brain mitochondria that likely lead to neurodegenerative diseases. As well, the prior administration of the synthetic antioxidant, NAC, could prove beneficial in combating the MX adverse effects.

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الملخص العربي

تدهور محتوى السيتوكروم سي واختلال وظائف الميتوكوندريا في مخ ذكور الجرذان بعد التعرض شبه المزمن للثياميثوكسام والدور الوقائي لـ أن أستيل سيستئين

رضا خميس عبد الرازق ، إيمان محمد مسلم ، نادية على حامد

تحافظ الميتوكوندريا على وظائف المخ. تهدف هذه الدراسة لتقييم تأثير الثياميثوكسام (MX)) على ميتوكوندريا مخ الجرذان بالإضافة إلى الدور الوقائي لـ أن اسيتيل سيستئين ضد التأثيرات الضارة لـ MX. تم اعطاء MX للحيوانات بتركيز ٣١,٢٦ ملجم / كجم من وزن الجسم (٥٠/١ من الجرعة المميتة لـ ٥٠ % من الأفراد المعاملة) وذلك عن طريق الفم بخمس جرعات كل أسبوع لمدة ٢٨ يوم. أظهرت النتائج أن MX له سمية عصبية والتي تم تأكيدها من خلال الارتفاع المعنوى فى نشاط كل من انزيم الأسيتيل كولين استيريز واللاكتات ديهيدروجينز في بلازما الدم. كذلك حدث زيادة كبيرة في مضادات أكسدة الميتوكوندريا مثل السوبر أوكسيد ديسميوتيز والجلوتاثيون المختزل. أيضًا، أدت المعاملة بالمبيد الى تحفيز الإجهاد التأكسدي وذلك بزيادة مستويات المؤشرات الحيوية لاكسدة الدهون مثل المالونالدهيد وتلف الحمض النووي مثل ٨- هيدروكسى -٢ ديأوكسى جوانوزين للميتوكوندريا بنسبة ٣٢,٥٪ و ١١٨,٦١٪ على التوالي مقارنة بالمجموعة الضابطة. كما اظهرت النتائج انخفاض معنوى في نشاط انزيمات الناد ديهيدروجينز، السيتوكروم سى ريدكتاز، والسيتوكروم سي أوكسيديز، الأدينوزين ثلاثى الفوسفاتيز. بالإضافة إلى حدوث ٢٣٪ احتشاء دماغي من خلال التقييم النسيجي باستخدام مؤشر نشاط أنزيم الناد ديهيدروجينز وهو عبارة عن صبغة ٢ ، ٣ ، ٥- ثلاثي فينيل كلوريد المتخصصة لنشاط الميتوكوندريا فى النسيج. وخلصت الدراسة الى أن: - مبيد الثياميثوكسام يمكن أن يشكل خطرًا على سلامة وعمل النظم العصبية للجرذان (الثدييات) حيث أن انزيمات اصلاح التلف الجينى فى الميتوكوندريا ضعيفة جدا. بالإضافة إلى ذلك، قد يكون العلاج المبكر بمضادات الأكسدة الاصطناعية، أن اسيتيل سيستئين، مفيدًا في مكافحة الآثار الضارة لمبيد الثياميثوكسام.

الكلمات الدالة: المخ ، الناد ديهيدروجينز ، السيتوكروم سى ، تلف الحمض النووي ، نيونيكوتينويد.