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Neurotoxicity and Oxidative Damage Induced from Exposure of Male Rats to Low doses of the Synthetic Environmental Contaminant Tributyltin

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ABSTRACT: Tributyltin chloride (TBT) is an organotin compound widely used in several high biocides for agro-industrial applications, such as fungicides, and marine antifouling paints leading to endocrine disrupting actions, such as imposex development in mollusks. The current study investigates DNA damage, changes in brain drug-metabolizing enzymes, lipid profile, and oxidative damage of TBT at low doses 10, 100, 250, 500, 1000, and 2000 µg/kg B.W. in male rats. The animals were divided into eight equal groups which treated daily by oral gavage for 45 days. Administration of TBT induced a dose-dependent increase in the DNA damage and TBARS. Also, it decreases the expression of the regulator of the antioxidant response (the levels of glutathione and the activities of GR, GPX, CAT, and SOD). TBT did not show any significant effect of brain drug-metabolizing enzymes (cytochrome b5 and cytochrome p450). TBT at 250, 500, 1000, and 2000 µg/kg B.W. showed significant increase in lipid profile (total cholesterol, triglyceride, and LDL), but HDL showed significant decrease. In conclusion, the obtained results provide evidence that exposure to TBT at low doses causes oxidative stress, DNA damage, and changes in biochemical parameters in a dose dependent manner.

Keywords: Tributyltin chloride; Neurotoxicity; Molecular parameters; Oxidative stress; Drug-metabolizing enzymes; Lipid profile.

1. INTRODUCTION

Organotin compounds have a broader range of technological and industrial applications than any other metal's organic compounds **(1)**. Organotin compounds (OT), especially tributyltin (TBT), have been used since 60 s in fungicides, farming and industrial biocides, plastics stabilizers and specially employed in antifouling paints. The most extensively studied organotin is tributyltin (tributyltin which is used as a plastic stabilizer and in broad-spectrum biocides (for example, agricultural fungicides**) (2)**. Tributyltin (TBT) and its metabolites, including dibutyltin and monobutyltin, are easily released into the environment as a result of incorrect disposal. Tributyltin builds up in the environment, then accumulates at various levels of the food chain **(3).**

Tributyltin can enter the aquatic ecosystem through the water column, sediments, and the consumption of infected food, exposing organisms to it. Tributyltin-contaminated sediments and consumption of tributyltin-contaminated food or water both have the potential to expose organisms in the terrestrial environment **(2).**

Tributyltin poses a significant threat to ecosystems. Even at low concentrations, tributyltin chloride compounds are highly toxic to a wide range of aquatic organisms. Tributyltin chloride is

especially dangerous due to the fact that it builds up in both these organisms and the mammals and fish that eat them **(4)**.

Tributyltin increased cortisol levels and caused disruption in neurotransmitter pathways (dopamine and gamma-aminobutyric acid, serotonin) **(5).** Tributyltin chloride can alter chemical neurotransmitters and disrupt the blood-brain barrier. As a result, the brain is being investigated as a possible target for tributyltin toxicity **(6).** Furthermore, tributyltin inhibits ATP synthase, which is in charge of ATP synthesis in the mitochondria. Inhibitors of this enzyme are thus potentially harmful to all life forms. Tributyltin chloride also prevents ATP hydrolysis by inhibiting ATPases **(7).** Ttiphenyltin (TPT) and TBT are the only 2 amongst the 67 endocrine-disrupting compounds scheduled by the US Environmental Protection Agency and consider toxic to the aquatic environments **(8)**.

TBT is suggested to stimulate the generation of reactive oxygen species (ROS) inside cells (Ishihara et al., 2012). Under normal circumstances, the brain maintains the balance between pro- and antioxidants. When such balance is distressed, neuropathologies develop **(9).** Numerous neurodegenerative disorders, including Alzheimer's disease (AD), multiple sclerosis, and Parkinson's disease (PD), have been associated with the oxidative stress. Lipid peroxidation is those outcomes of ROS attacking lipids, Protein modification causes the malfunctioning of enzymes like glutamine synthase and superoxide dismutase (SOD). Apoptosis and neuronal cell death are caused by ROS's ability to promote glutamate receptor excitotoxicity through an excess of calcium **(10)**.

2. MATERIALS AND METHODS

2.1. Tested compounds and doses

Tributyltin chloride ($C_{12}H_{27}ClSn$) purchased from Sigma–Aldrich Chemical Company, St. Louis, MO, USA, in the form of clear liquid, and light yellow. Product No: T50202-5G, CAS- 1461-22-9, purity 96% and Molecular Weight: 325.51 g/mol. All reagents and chemicals were of analytical grade. The doses of tributyltin chloride were 10µg, 50µg, 100µg, 250µg, 500µg, 1000 µg, and 2000 µg/kg B.W. The doses were chosen according to **(6)**.

2.2. Animals and experimental design

Forty male Albino rats weighing 175 ±5 g (12 weeks old) were used in the present study. Animals were obtained from Faculty of Medicine, Alexandria University, Alexandria, Egypt. The research was carried out according to the Guide for the Care and Use of Laboratory animals (International Council for Laboratory Animal Science, ICLAS) and was approved by the local ethical guidelines of Institutional Animal Care & Use Committee (IACUC), Alexandria University, Egypt (AU14-211017-2-8) and all the methods were performed according to the guidelines and regulations of the same Committee. Rats were kept on basal diet and tap water is provided *ad libitum*. Animals were kept in normal atmospheric condition at a temperature of 25 ± 5 °C and 50-70% humidity were maintained throughout the experiments with a 12 h light/day cycle. After two weeks of acclimation, animals were randomized divided into 8 equal groups (5 rats per each group): group1 served as control and received vehicle dimethyl sulfoxide (DMSO) at dose 1µL /1 g bw rat; groups 2, 3, 4, 5, 6, 7 and 8 were treated with TBTC at of 10µg, 50µg, 100µg, 250µg, 500µg, 1000µg, and 2000 µg/kg B.W., respectively. Animals were orally gavaged daily with respective doses for 90 consecutive days.

2.3. Blood samples collection and tissue preparations

Animals were sacrificed by the end of the experimental period using isoflurane 100 % (2 mg/Kg BW or equal 2% inhaled in dictator). Blood samples were collected by cardiac puncture and centrifuged for the separation of plasma (860 xg for 20 min) that was kept at -80 ◦C until analysis. The plasma was kept at - 80 ˚C until analyses of the tested parameters. Brain was immediately

removed, washed using chilled saline solution (0.9 %), and removed the adhering fat and connective tissues. Brain was minced and homogenized (10 %, w/v), separately, in ice-cold sucrose buffer (0.25 M) in a Potter–Elvehjem type homogenizer. The homogenates were centrifuged at 10,000 xg for 20 min at 4 ◦C, to pellet the cell debris and the supernatant was collected and stored at -80 ◦C for the determination of tested parameters.

2.4. Markers of oxidative stress and antioxidant parameters

Malondialdehyde (MDA), a lipid peroxidation index in the brain homogenate, was assayed as thiobarbituric acid-reactive substances (TBARS) using the method of (Tappel and Zalkin (1959). Glutathione peroxidase (GPx), Glutathione S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD) activities were assayed according to Chiu et al. (1976), Habig et al. (1974), Luck (1974) and Misra and Fridovich (1972), respectively. Reduced glutathione (GSH) content was determined, and the method utilized metaphosphoric acid for protein precipitation and 5, 5′ -dithiobis (2-nitrobenzoic acid) for color development that was measured at 412 nm **.** Glutathione reductase (GR) was determined according to the method described by **(11)** by the kits obtained from Bio diagnostic, Egypt. Reduced glutathione (GSH) was determined according to the method described by **(12)** by the kits obtained from Bio diagnostic, Egypt.

2.5. Determination of DNA breakages in brain homogenate

DNA fragmentation % assay was conducted using the procedure of **(13)**. The tissue (50 mg) was homogenized in 10 volumes of a TE solution pH 8.0 (5 mmol/L Tris–HCl, 20 mmol/L EDTA) and 0.2% triton X-100. 1.0 ml aliquot of each sample was centrifuged at 27,000 × g for 20 min to separate the intact chromatin (pellet, B) from the fragmented DNA (supernatant, T). The pellet and supernatant fractions were assayed for DNA content using a freshly prepared DPA (Diphenylamine) solution for reaction. Optical density was read spectrophotometry at 620 nm. The results were expressed as amount of % fragmented DNA by the following formula:

Fragmented DNA (%) = T X 100/ (T + B)

T: Measure absorbance of Supernatant

B: Measure absorbance of Pellet

2.6. Assay of cytochrome b_5 in brain

Brain total cytochrome b5 was assessed using the method of **(14).** The brain homogenate (0.5mL) has been diluted with (4.5mL) 0.1 mol/L phosphate buffer (pH 7.40), and a few crystals of sodium dithionate. Following mixing, the binding spectrum has been recorded spectrophotometrically over wave lengths 450 nm, with base line subtraction. With the use of an extinction value of 185 $cm^{-1}mmol^{-1}$, the concentration regarding reduced cytochrome b5 was determined.

Cytochrome b_5 concentration (nmol cytochrome/mg protein) = $\left(\frac{A}{\varepsilon}\right)$/mg protein

ε = Molar extinction coefficient 185 $mmol^{-1}$/cm.

A = Absorbance of sample

2.7. Assay of cytochrome P450 in brain

Brain total cytochrome P_{450} (CYP_{450}) was assessed using the method of **(14)**. The brain homogenate (0.5 mL) has been suspended in 4.5mL of 0.1mol/L phosphate buffer (pH 7.4) and reduced by adding a small amount of sodium dithionate crystals. After this, carbon monoxide has been bubbled for one minute at a rate of 20–30 bubbles in sample. After that, using spectrophotometry at wavelengths of 450 nm, the decreased CYP_{450}-CO complex was recorded. The extinction coefficient of 91 $Cm^{-1}mmol^{-1}$ was used to calculate the concentration regarding the hemoprotein-CO complex.

Cytochrome P450 concentration (nmol cytochrome/mg protein) = $\left(\frac{A}{\varepsilon}\right)$/mg protein

ε = Molar extinction coefficient 91 $mmol^{-1}$/cm.

A = Absorbance of sample

2.8. Lipid profile

The parameters of the plasma lipid profile (Triglycerides; TG, total plasma cholesterol, High-density lipoprotein-cholesterol; HDL-C, and Ligh-density lipoprotein-cholesterol LDL-C) were assayed using commercially available kits (Biosystem, Spain) **(15)**.

2.9. Statistical analysis of the data

Mean and standard error values were determined for all the parameters and the results were expressed as mean ± standard error. The data were analyzed using a one-way analysis of variance (ANOVA) followed by Duncan multiple comparison. $P<0.05$ was statically significant according to **(16)**.

3.RESULTS AND DISCUSSION

The results revealed that total cholesterol and triglycerides insignificantly changed in the groups treated with 10 and 50 µg /kg bw of TBTC, while 100, 250, 500, 1000, and 2000 µg/kg caused significant increase in these parameters compared to control group. LDL-C insignificantly changed in the groups treated with 10, 50, and 100 µg /kg bw, while significantly increased in the reset of treated groups (250, 500, 1000, and 2000 µg /kg bw) compared with control groups. On the other hand, HDL-C did not show significant changes in all treated groups **(Fig 1).**

The increase in lipid profile in the present study was like the obtained results of the study of **(17)** who found that TBT exposure increased total cholesterol and triglyceride levels significantly in both male and female rats. **(6)** reported that reduced lipoprotein lipase (LPL) activity may underlie TBTC's hyperlipidemia impact and increased the accumulation of interstitial ectopic lipids. These indicators were elevated by sub-chronic TBTC exposure and may be contribute to the development of coronary artery disease. Also, **(18)** found that exposure to TBT increased adipose tissue formation and subsequent obesity**.**

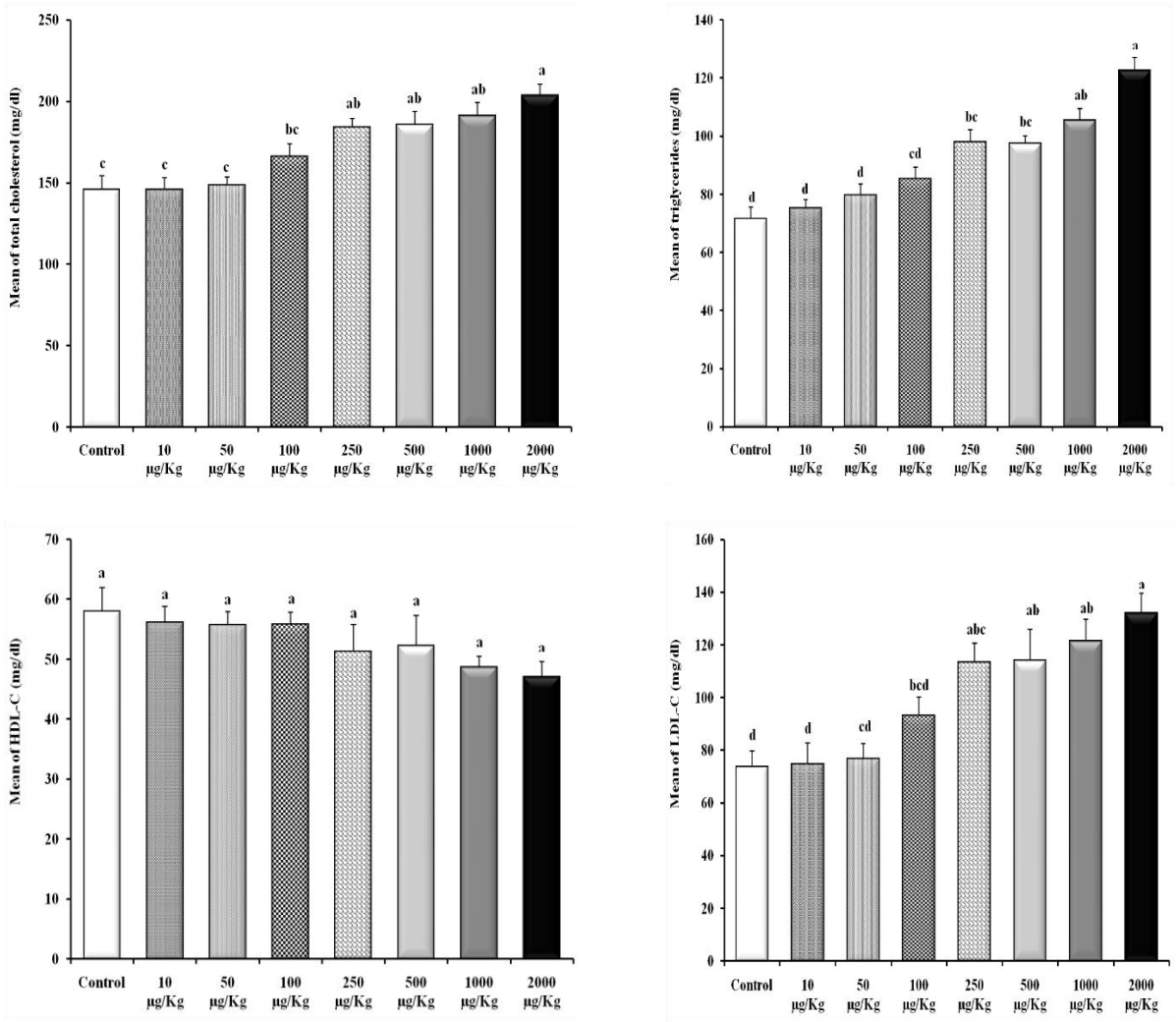


Figure1: Changes in plasma total cholesterol, triglycerides, HDL-cholesterol, and LDL-cholesterol of male rats treated with tributyltin chloride.
Value expressed as means ± S. E; n = 5 rats each group.
Superscript letters (a, b, c, d, e) are significantly different at $p < 0.05$.
Mean with letter a is the highest one, followed by another letter, then e is the lowest one

The obtained results showed that DNA breakages insignificantly changed in the treated with 10 and 50 µg /kg bw TBTC, and significantly increased in groups treated with 100, 250, 500, 1000, and 2000 µg/kg of TBTC in a dose dependent response when compared with the control group **(Fig 2).** Results showed that GSH insignificantly changed in the groups treated with 50, 100 and 250 µg /kg of TBTC. Meanwhile, GSH significantly decreased in the groups administered with 500, 1000, and 2000 µg /kg of TBTC in a dose dependent response when compared to control group **(Fig 2).**

In the hippocampus slices, **(19)** discovered that nucleosome DNA fragmentation happened after TBT-induced ROS generation**. (20)** found that TBT caused apoptosis or DNA damage makers (procaspase-3, caspase-6, and PARP cleavage), significantly delayed and decreased. After 90 min of treatment at 2 µM, TBT preferentially promotes DNA fragmentation without signi¢cant loss of plasma membrane integrity, whereas at 2.5 µM, TBT causes a signi¢cant loss of viability with only a weak DNA ladder **(21).** In general, ROS triggers several intracellular processes that all result in cell damage, such as kinase signalling, mitochondrial malfunction, and/or caspase activation, (**22**). Furthermore, researchers noted significant oxidative stress decreased permeability of mitochondrial membrane that was accompanied by nucleosome DNA breakage induced by endonuclease G, which

had been transferred from the mitochondria towards the nuclei. Also, the present results showed that TBT induced ROS and oxidative damage

The glutathione system is crucial for shielding healthy brain tissue from oxidative damage. At the level of redox status of brain tissues, the TBTC induced a significant reduction in the main anti-oxidant systems in the brain GSH systems. GSH levels have been depleted considerably, especially with the higher doses of TBT **(23)**. TBT may readily interact with the free-SH group in the antioxidant proteins such as glutathione and SOD reducing their action against stress conditions **(24)**. In line with this study, **(25)** reported that cortical tissues may experience oxidative stress as demonstrated by significant ROS production and GSH deficiency may be through trace element imbalance and the direct inhibitory impact of TBTC upon glutathione-S-transferase. **(26)** showed that TBT may penetrate numerous areas of the brain increasing ROS levels and inducing alterations in GSH levels, particularly in the striatum as well as the cerebral cortex.

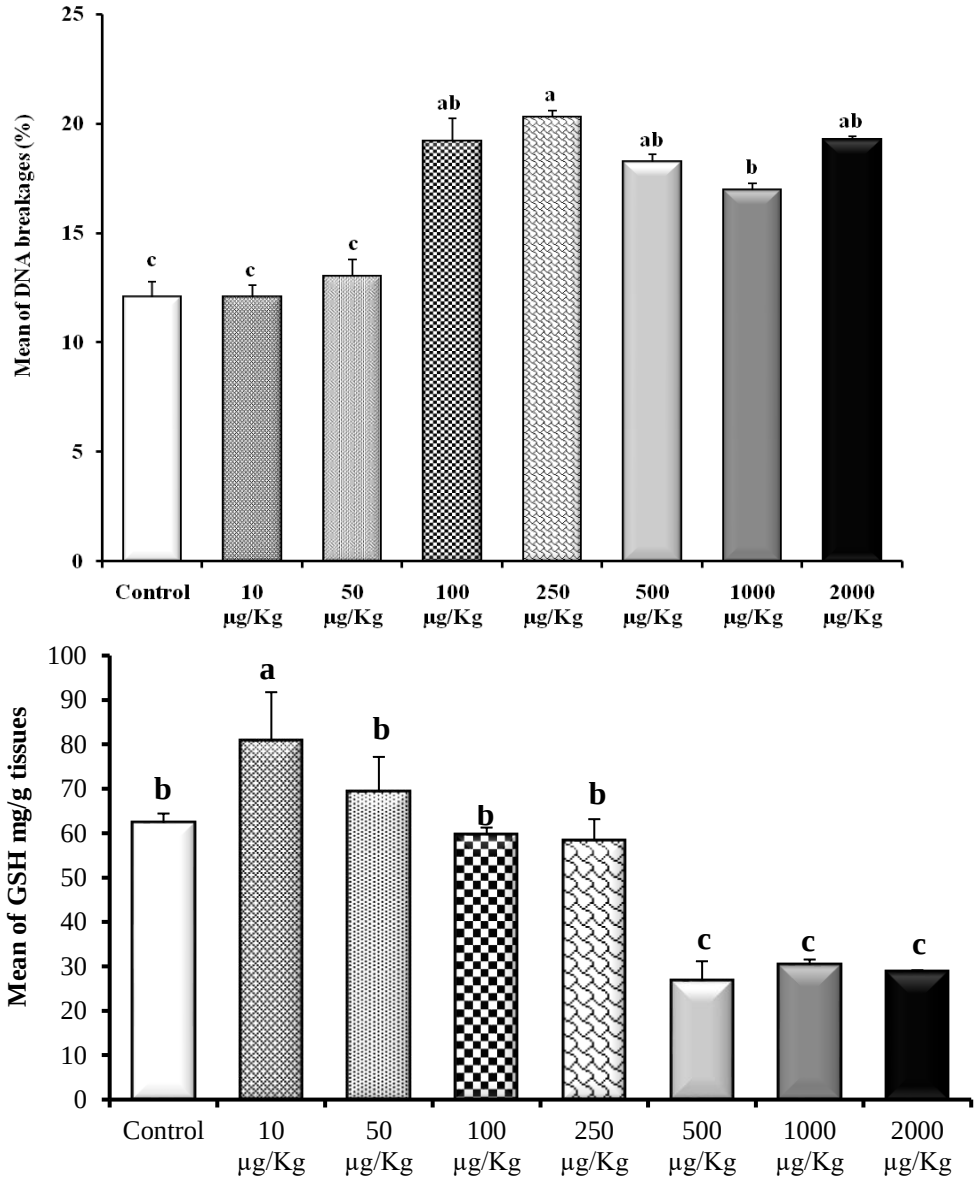


Figure 2: Changes in the reduced glutathione and DNA Fragmentation in the brain of male rats that have been supplemented with tributyltin chloride

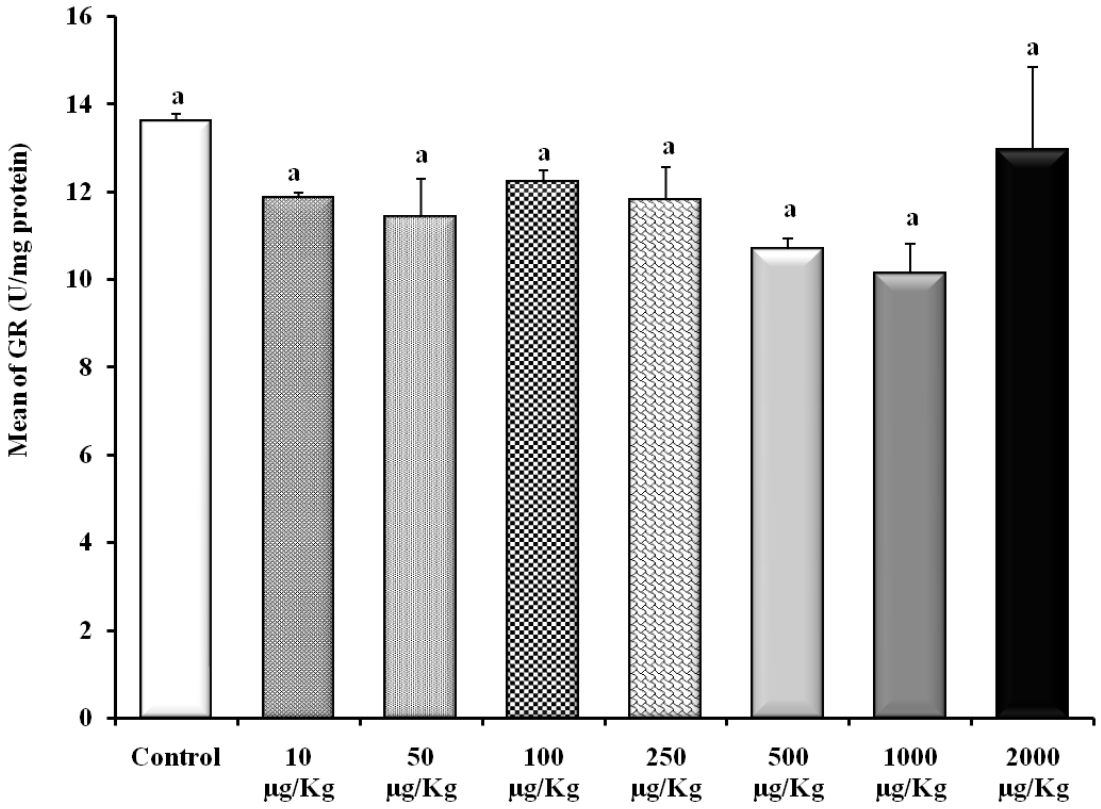
Value expressed as means ± S. E; n = 5 rats each group

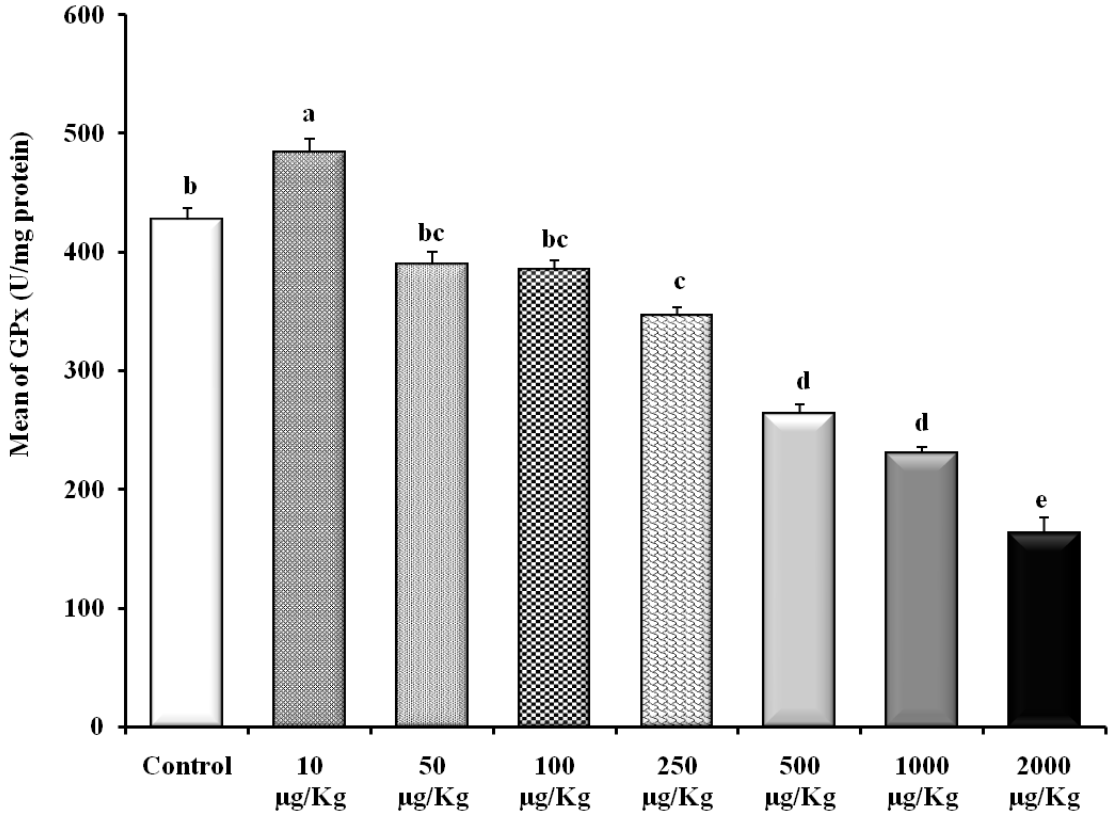
Superscript letters (a, b, c, d, e) are significantly different at $p < 0.05$.

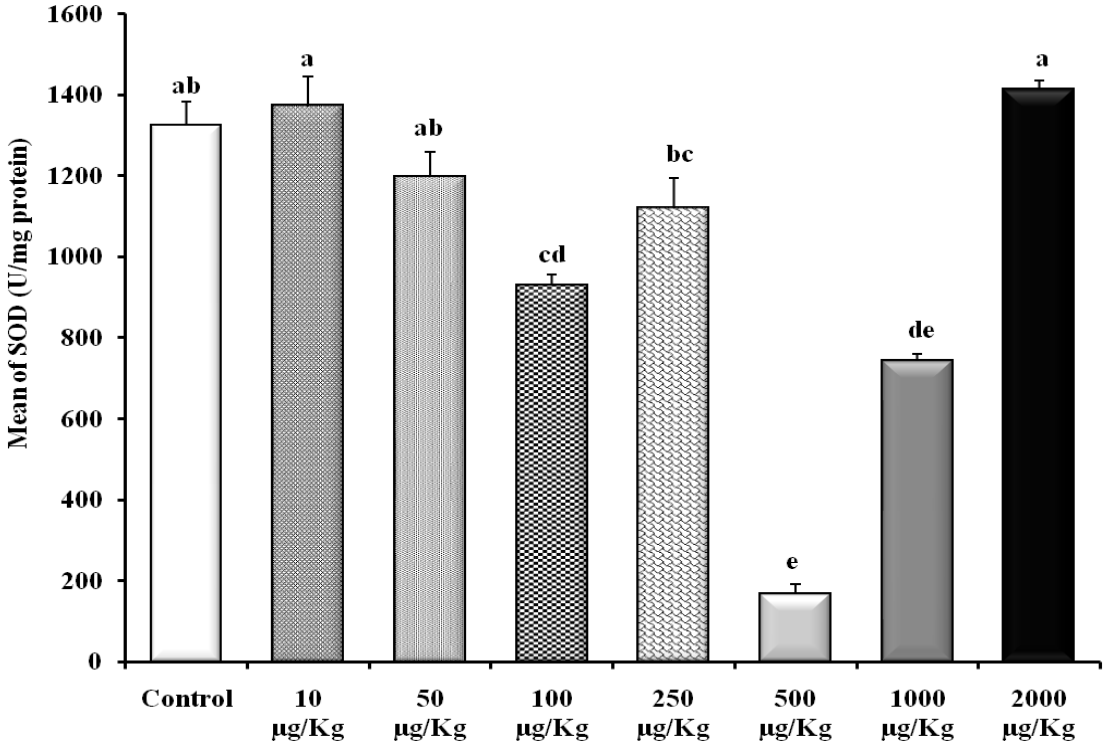
Mean with letter a is the highest one, followed by another letter, then e is the lowest one

Results showed that TBTC caused significant decreases in the activities of brain glutathione peroxidase (GPX), superoxide dismutase (SOD), glutathione reductase (GR), and catalase (CAT). The increase of the dose of TBTC significantly decreases activities of antioxidant enzymes to reach the lowest activity at the highest dose (2000 µg/kg) compared with the control group **(Fig 3).**

Antioxidants have an impact on protecting biological macromolecules from oxidative damages. However, when antioxidant defences are compromised, free radicals can cause biological membrane oxidation, leading to cellular instability and excess MDA formation. As indicated also by MDA concentrations, however, the oxidative stress can be mitigated through antioxidant enzymes at very low levels of TBTC **(27).** Also, there was a decrease in CAT, SOD, and GPx levels in the zebrafish liver of those exposed to 10-100ng/L TBTC, as it has been reported by **(28).** According to **(23) ,** TBTC significantly changed several enzymes, particularly GST, GR, SOD, GPX, and CAT . Inhibiting glutathione-S-transferase can impair the synthesis of GSH conjugates, one of the crucial steps in detoxifying xenobiotics **(23)**.




Mean of GPx (U/mg protein)
600
500
400
300
200
100
0
b
a
bc
bc
c
d
d
e
Control
10 µg/Kg
50 µg/Kg
100 µg/Kg
250 µg/Kg
500 µg/Kg
1000 µg/Kg
2000 µg/Kg


Mean of SOD (U/mg protein)
1600
1400
1200
1000
800
600
400
200
0
ab
a
ab
cd
bc
e
de
a
Control
10 µg/Kg
50 µg/Kg
100 µg/Kg
250 µg/Kg
500 µg/Kg
1000 µg/Kg
2000 µg/Kg

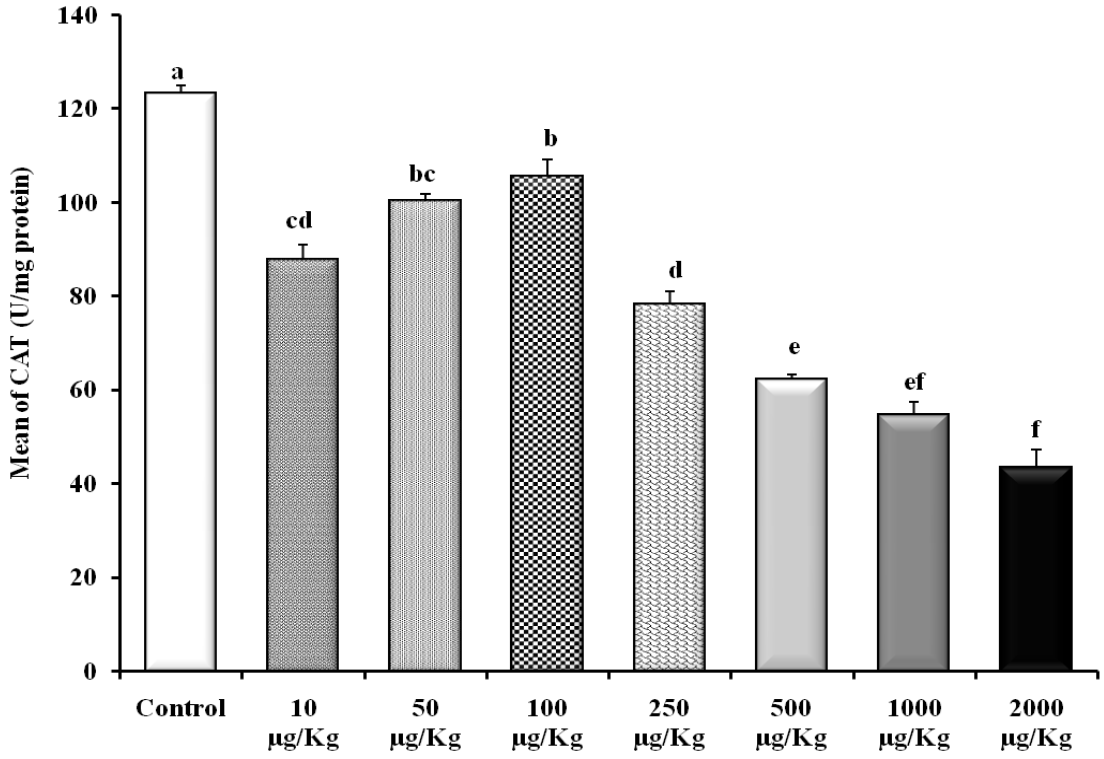


Figure 3: Changes in enzymatic antioxidants; GR, Gpx, CAT, and SOD in the brain of male rats that have been supplemented with tributyltin chloride
Value expressed as means ± S. E; n = 5 rats each group.
Superscript letters (a, b, c, d, e) are significantly different at $p < 0.05$.
Mean with letter a is the highest one, followed by another letter, then e is the lowest one

The results showed that Cytochrome P450 changed in the groups treated with TBT at doses of 10, 50, 100, 250, 500, 1000, and 2000 µg /kg of TBTC. Meanwhile, Cytochrome b5 insignificantly changed in all groups treated with TBTC, except the group treated with 2000 µg /kg showed significant increase **(Fig 4).** Variations in CYPs activity might have a major impact on a person's health because CYPs are a key enzyme for such detoxification by foreign substances and could be utilized as indications of the ecological damages and for the monitoring of the environment **(29)**. TBT can combine using microsomal CYP to influence its P450 process in marine environments **(30**). Changes in CYP450 activity have a significant impact on the capacity for xenobiotic activity as well as detoxification and this is significant in terms of toxicology **(31)**. In fresh-water fishes, **(32)** showed that TBT could engage with microsomal CYP450 both *in vitro* and *in vivo*, inactivating ethoxyresorufin-O-deethylase (EROD) enzymatic reaction concentrations. TBT might have an impact on aquatic species' P450 networks. It was established whether cytochrome P450 is involved in the potential metabolic mechanism for the formation of imposex. This could be a factor in mollusks' sensitivity to TBT ingestion **(33)**. Changes in these markers showed that TBT exposure altered regulation of central nervous system in zebrafish (**34).**

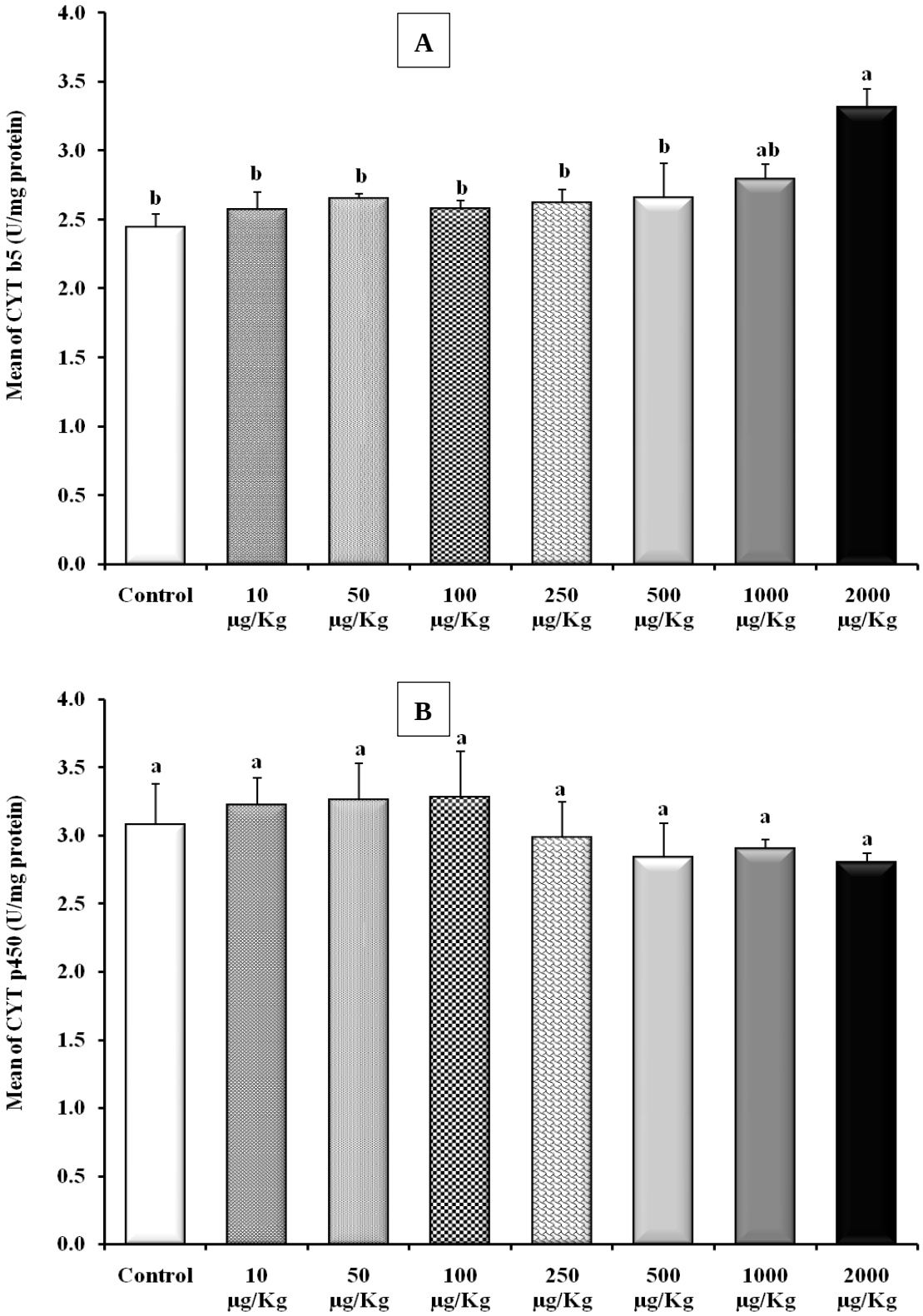


Figure 4: Changes in Cytochrome b5 (A) and Cytochrome P450 (B) in the brain of male rats that have been supplemented with tributyltin chloride

The results were represented in the form of Mean ± SE, n=5.

Mean values in same column sharing a shared subscript letter were not significantly different, while those with different subscript letters were significantly different. $p < 0.05$.

CONCLUSION

In conclusion, the obtained results provided solid evidence that neurotoxicity induced by TBTC at low different doses (10, 100, 250, 500, 1000, and 2000 µg/kg B.W.) through changes in DNA damage, drug-metabolizing enzymes, lipid profile, and oxidative damage of TBT at low doses 10, 100, 250, 500, 1000, and 2000 µg/kg B.W. This study suggests further investigation on the lowest doses of TBT for different time of exposure.

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