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NOS Expression in Oxidative Stress, Neurodegeneration and Male Infertility Induced by the Abuse of Tramadol

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Abstract

Tramadol is widely used as a pain reliever; stimulant and it may delay ejaculation. But chronic tramadol users have adverse side effects.

Aim: To clarify the worst effect of tramadol hydrochloride on male fertility and its role in neurodegeneration disorders due to oxidative stress in spite of indeed its use as ejaculation delaying.

Materials and methods: This experimental study was performed upon 30 adult wistar rats. They were purchased from the National Research Centre Cairo, Egypt. These rats were divided into three groups (n=10), Group I, rats received vehicle and served as normal control. Group II, rats received low dose of tramadol (20 mg/Kg body weight/day). Group III, rats received high dose of tramadol (50 mg/Kg body weight/day) for 12 weeks.

Results: The results illustrated that tramadol in both low and high doses ingestion lead to weight loss, elevated Nitric Oxide Synthase (NOS) expression and plasma peroxidation value (MDA) but reduced Glutathione (GSH) and Superoxide Dismutase (SOD) in plasma were significantly decreased. Furthermore, plasma Monoamine Oxidase (MAO) was increased leading to serotonin (5-HT) and dopamine decrease in brain tissues proteins ($p<0.001$). Plasma Testosterone level, sperm content and sperm vitality were significantly decreased ($p<0.001$). Furthermore, hematoxylin and eosin (H&E) stain of the brain tissues showed few purkinje cells and the cells of granular layer were widely separated in the rats of low dose of tramadol group, but there was shrunken purkinje cells, some cells had pykinotic nuclei and majority of the cells of granular layer were non-nucleated, few of them had pykinotic nuclei in the rats of high dose of tramadol when compared to normal brain tissues of normal control rats.

Conclusion: Long term tramadol administration developed oxidative stress, neurodegenerative disorders and male infertility.

Keywords: Neurotransmitters; Tramadol; Serotonin; Expression; Glutathione

Introduction

Tramadol is one of the most common causes of poisoning in adult male patients with the previous history of drug addiction and psychological problems. Suicide is the most common motivation for these patients [1].

Tramadol is a synthetic analogue of codeine with central effects. It is not an opioid derivative or Non-Steroidal Anti-Inflammatory Drug (NSAID) medication. Actually, tramadol has low affinity for opioid receptors [2].

Tramadol is conducted orally, rectally, extended release and intramuscular/intravenous solution. The administration of tramadol orally has rapid absorption, distribution and maximum concentration in blood. It is able to pass the placental barrier and it may be detected in breast milk [3].

The common therapeutic dose of tramadol is 50 to 100 mg (50 mg oral, 50-100 mg Intramuscular (IM), and/or100 mg rectal; 1.5 mg/kg/day in a 60-kg patient) three to four times a day. Doses higher than 400 mg/day are generally not necessary.

Tramadol is used as the first-line therapy in muscles and skeletal pains and as a main treatment for osteoarthritis in cases of contraindication for NSAIDs and for pain resistance for the other analgesics. According to a study performed on the rats, tramadol reinforces the immune system by increasing phagocytosis. Use of tramadol is therefore favored as an analgesic in immunocompromised patients [4].

Tramadol is an analgesic with fewer side effects in comparison with other opioids. It has the least gastrointestinal and renal toxicities. Dizziness, nausea, constipation, vertigo, and headache are the most common symptoms [5].

Tramadol is a centrally acting analgesic prescribed off-label for the treatment of Premature Ejaculation (PE). However, tramadol may cause addiction and difficulty in breathing.

Tramadol is neuroprotective and relieves the cerebral injuries created by hind limb ischaemia–reperfusion. Toxicity from tramadol

appears to be due to monoamine uptake inhibition rather than its opioid effects [6].

Tramadol reduces plasma levels of Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), total cholesterol, but elevates prolactin and estradiol levels. Also, tramadol increases the testicular levels of nitric oxide, lipid peroxidation and decreases the antioxidant enzymes activities. Tramadol can exaggerate the endothelial nitric oxide synthase expression in testicular texture [7].

Oral administration of tramadol decreases the neurotransmitter levels [8]. Also, many described tramadol as having antidepressant-like effects. However, serotonin syndrome may be developed as adverse reactions during misuse/overdose tramadol monotherapy. This syndrome is most often the result of a prescription drug, overdose of causative drugs, and/or complex interactions among several drugs. Three key clinical features of this syndrome are as follows: Neuromuscular hyperactivity (e.g., tremor, clonus, myoclonus, hyperreflexia, and rigidity), autonomic hyperactivity (e.g., diaphoresis, fever, tachycardia, tachypnea, mydriasis, diarrhea) and altered mental status (e.g., agitation, confusion) [9].

Oxidative stress is an imbalance between the Reactive Oxygen Species (ROS) formation and the ability of the biological system to neutralize them with enzymatic and/or non-enzymatic antioxidant. A large number of reactive intermediate may induce cell damage with the production of secondary toxic compounds, such as aldehydes and ketones, thus causing an elevated risk of a lot of diseases. There are a number of participating in the elimination of free radicals and other ROS systems produce, but these mechanisms are not fully effective [10].

The respiratory process is the source of manufacturing of reactive oxygen species, with modified cellular function. ROS include extremely reactive species as oxygen ions, free radicals, and organic and inorganic peroxides. ROS is related to harmful cellular effects, such as DNA damage, polyunsaturated fatty acids oxidation and amino acids oxidation. Peroxyl radicals (ROO.), hydroxyl radicals (HO.), superoxide ion (O^{2-}), and singlet oxygen ($1O_2$), are leading factors for a lot of diseases as aging, cancer and Alzheimer's disease. Also, tramadol can attenuate the antioxidant capability [11].

Oxidative stress damage may affect the brain especially, as the brain can diminish a lot of oxygen particles and generate plenty of free radicals. ROS is very toxic to cellular homeostasis [12].

Alzheimer's Disease (AD) is a CNS neurodegenerative disorder with exaggerated loss of cognition. Neurotoxic β-amyloid peptide (Aβ) if increased in the brain, it means that there is a neuropathological lesion in the brain of patients with AD [13].

Normally, mitochondrial respiratory metabolism can develop toxic ROS but cellular antioxidant defense mechanisms can repair it. The imbalance between the ROS production and cellular antioxidant defense creates the cellular destruction. Therefore, the formed oxygen free radical products can form covalent binding with free sulfhydryl group forming proteins in soluble brain extract such as actin and "serotonin binding proteins" [14].

Monoamine Oxidase (MAO) enzymes were proposed and designated MAO A and B. MAO A has higher affinity for the substrates serotonin, norepinephrine, dopamine, and the inhibitor clorgyline, whereas MAO B has higher affinity for phenylethylamine, benzylamine, and the inhibitor deprenyl [15].

MAO itself is an H_2O_2 generating enzyme. Therefore, the increased turnover of dopamine neurotransmitter and its O-methylated metabolite (3-O-methyl-Dopamine) can be looked upon as an endogenous oxidative stress, increasing the steady state level of H_2O_2 and evoking oxidation of GSH to oxidized glutathione (GSSG) both pre- and post-synaptically in the region of dopamine neurons and nerve terminals [16].

Tyramine + O_2 + H_2O → H_2O_2 + NH_3 + p-hydroxyphenylacetaldehyde

Oxidative stress exaggeration is the mechanism of the various tissues injury including testis. Increased oxidative stress leads to testosterone decline can damage testosterone secreting Leydig cell. Oxidative stress is one of the causes of male infertility. Damage of lipid, proteins, carbohydrates and DNA is a leading factor for the significant oxidative damage to many cellular organelles which occurs mainly due to the excess production of ROS more than the antioxidant defence, which may be a leading cause for cell death. Sperm contains high poly unsaturated fatty acid, so is susceptible to oxidative damage [17].

Materials and Methods

Experimental design

This experimental study was carried out on 30 adult healthy Wistar rats weighing 190-200 g. They were obtained from the National Research Centre Cairo, Egypt. All rats had free access to standard chow diet and tap water. They were housed under control condition at temperature (25°C) with 12 h light/dark cycle. Rats were divided into three groups (10 in each). 1st group was normal control, 2nd group was supplemented with low dose of tramadol hydrochloride (20 mg/kg body weight/day orally for 12 weeks). 3rd group was supplemented with high dose of tramadol hydrochloride (50 mg/kg body weight/day orally for 12 weeks).

Plasma collection and biochemical analysis

At the end of the experiment, the rats were fasted overnight and the blood was obtained via retro-orbital bleeding [18]. The blood was collected in EDTA-coated glass tubes, and centrifuged at 1000 × g for 15 min at 4°C. The plasma were collected and stored at -70°C for estimation of MAO, MDA, SOD and testosterone hormone assays. Peroxidation (MDA) was determined spectrophotometrically as described by Jain [19] and Janero [20]. SOD according Jemec et al. [21]. MAO enzyme was measured using Rat MAO ELISA Kit from Bio Assay Systems/USA [22]. Determination of GSH using Rat GSH ELISA Kit, El-Aab, Catalog No: E0294r. The plasma testosterone concentration was determined using (ELISA; DRG Instruments GmbH, Marburg, Germany). All dry chemicals and solvents were obtained from Sigma Chemicals.

Preparation of brain Homogenate

Whole brain tissues were removed quickly on ice and homogenized. Determination of serotonin, dopamine levels were assayed in the brain homogenate. Also, NO synthase expression was assayed in the homogenate. Whole brains were washed in phosphate-buffered saline. Homogenizing solution (20 mmol/l HEPES, pH 7.5 with 0.1 mmol/l EDTA, 1 mmol/l DTT and mammalian protease inhibitor cocktail) 2.5 ml per 0.5 g tissue was added to the samples. Homogenized tissue was transferred to 50 ml centrifuge tubes and centrifuged at 1000 g, 4°C for

20 min. Supernatant was decanted into fresh tubes and pellets were discarded. Then supernatant was centrifuged at 10000 g, 4°C for 20 min. Supernatant was analysed for NOS protein expression using Western blot analysis then quantitative reverse-transcription polymerase chain reaction was performed against 18 S ribosomal RNA as a control gene. RNA quantity was determined using 18 S rRNA primers/probes (Applied Biosystems) and NOS expression determined using the primer/probe sequences. NOS expression was determined by dividing the quantity of message of interest by the quantity of 18S ribosomal RNA.

Serotonin (5-HT) and dopamine were determined fluorometrically [23].

Primer sequences used for determination of NOS

Forward: TCTGCGGCGATGTCACTATG

Reverse: CATGCCGCCCTCTGTTG

Probe: CCAGCGTCCTGCAAACCGTGC

Body weight

The animals' weights were recorded every week with a digital scale. Also, weights were recorded at the beginning and at the end of the experimental period to determine the difference between these records.

Motility and sperm count

The left caudal epididymis was separated and the total recovered sperm during 4 h of incubation in normal saline (volume=1 ml, 3537°C) was calculated. The sperm concentration was determined by the conventional method using a haemocytometer chamber for RBCs count. The right epididymis was finely minced by anatomical scissors in 1 ml of warmed isotonic saline in a petri dish. The sperm motility was estimated by evaluating 4 fields of a sperm droplet under a cover-slip on a warm glass slide (3537°C) under light microscopy (×40). The sperm vitality was assayed using a conventional procedure of eosin B-nigrosin stain (1.67% eosin, 10% nigrosin, and 0.1 M sodium citrate) under ×100 magnification and 100 sperm were counted. All of the sperm evaluation procedures were carried out based on WHO manual for human sperm analysis [24] with some modifications.

Lowry's method for estimation of protein

Principle

Reaction of –CO-NH- bond (peptide) in polypeptide chain with $CuSO_4$ in an alkaline medium to give a blue coloured complex [25]. Reduction of the phosphomolybdate and phosphotungstate components of the Folin-Ciocalteau reagent by the effect of tyrosine and tryptophan residues of protein to give bluish products.

Histological studies

The selected paraffin blocks for histological staining were sectioned (2-µm thickness) and stained with hematoxylin and eosin (H&E) stain [26].

	Normal	Low dose of tramadol	High dose of tramadol
	Mean ± SEM	Mean ± SEM	Mean ± SEM
Body weight (g)	196 ± 1.08	169.6 ± 2.01*	157.8 ± 1.31*#
Sperm number (x106)	59.7 ± 1.5	46.7 ± 1.3*	32.7 ± 1.4*#
Sperm motility (%)	66.0 ± 2.9	51.6 ± 2.2*	37.2 ± 1.2*#
Testosterone (ng/mL)	4.31 ± 0.24	3.13 ± 0.14*	2.65 ± 0.17*#

Table 1: Effect of low and high doses of tramadol on body weight, sperm number, sperm motility and testosterone level. * Significantly different from normal group ($p<0.001$). #Significantly different from the low dose group of tramadol Hcl ($p<0.05$).

Body weight and morphologic parameters

Table 1 showed that the body weight was significantly decreased in both low and high doses of tramadol groups when compared to the normal group ($P<0.001$). Furthermore, there was a significant decline in the high dose of tramadol group when compared to the low dose of tramadol group ($P<0.05$).

Plasma testosterone

Also, the plasma testosterone concentrations were significantly decreased at low and high doses of tramadol supplementation when compared to the normal rats ($P<0.001$) (Figure 1). Also, there was a significant decrease in the group of the high dose of tramadol when compared to the group of the low dose ($P<0.05$).

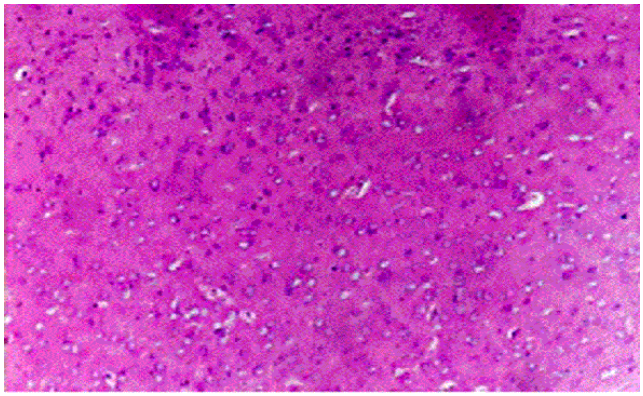

Figure 1: A photomicrograph of a section in brain of normal control (NC) rats.

Results

Sperm evaluation

Furthermore, the total sperm count and sperm vitality were significantly reduced at low and high doses of tramadol supplementation when compared to the normal rats (P<0.001). Also, there was a significant decrease in the group of the high dose of tramadol when compared to the group of the low dose (P<0.05).

	Normal	Low dose of tramadol	High dose of tramadol
	Mean ± SEM	Mean ± SEM	Mean ± SEM
5-HT (µg/g protein)	44.0 ± 1.5	28.4 ± 0.8*	18.7± 1.2*#
Dopamine (ng/g protein)	433.8 ± 4.2	327.9 ± 3.5*	221.8 ± 4.9*#
NOS (Expression)	3.74 ± 0.33	7.82 ± 0.34*	16.6 ± 0.90*#

Table 2: Effect of low and high doses of tramadol on serotonin (5-HT), dopamine and NOS concentration in brain tissues. *Significantly different from normal group (p<0.001). #Significantly different from the low dose group of tramadol Hcl (p<0.05).

Antioxidant activity

Table 2 recorded those NOS concentration was significantly increased in brain tissues of groups with low and high doses of tramadol when compared to the normal group (p<0.001). Also, there was a significant increase in the group of the high dose of tramadol when compared to the group of the low dose (P<0.05) (Figure 2).

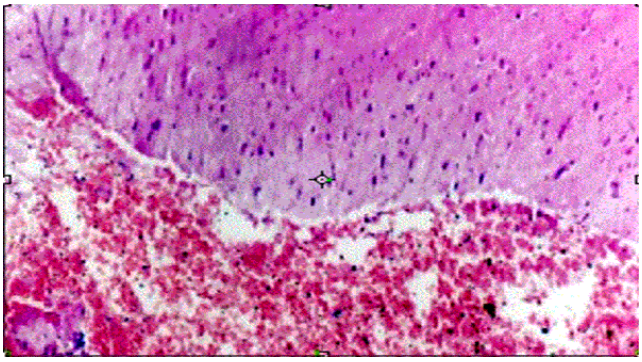

Figure 2: A photomicrograph of a section in brain of low dose of tramadol rats.

	Normal	Low dose of tramadol	High dose of tramadol
	Mean ± SEM	Mean ± SEM	Mean ± SEM
GSH (mg/ L)	45.5 ± 1.1	29.6 ± 1.4*	18.7 ± 1.0*#
MAO (uIU/mL)	59.2 ± 1.7	91.3 ± 2.1*	124.6 ± 2.7*#
MDA (nmol/L)	55.8 ± 2.1	87.8 ± 2.9*	114.6 ± 1.6*#
SOD (U/mL)	28.7 ± 1.3	18.7 ± 0.9*	13.4 ± 0.7*#

Table 3: Effect of low and high doses of tramadol on reduced glutathione (GSH), Monoamine oxidase (MAO), peroxidation value (MDA) and superoxide dismutase (SOD). *Significantly different from normal group (p<0.001). #Significantly different from the low dose group of tramadol Hcl (p<0.05).

Table 3 showed that, the level of SOD and GSH were significantly decreased in groups with low and high doses tramadol, while MDA level was significantly increased when compared to the normal group (p<0.001). Also, there was a significant decrease in the level of SOD and GSH in the group of the high dose of tramadol (Figure 3) when compared to the group of the low dose (P<0.05).

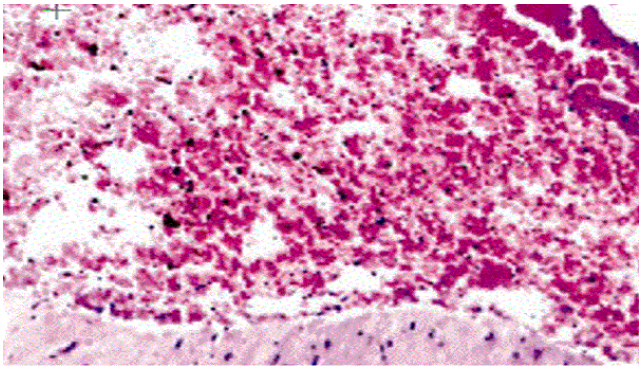

Figure 3: A photomicrograph of a section in brain of high dose of tramadol rats.

Neurotransmitters

Table 3 demonstrated that the plasma level of MAO was significantly increased in both groups with low and high doses of tramadol when compared to the normal group ($p<0.001$). Also, there was a significant increase in the group of the high dose of tramadol when compared to the group of the low dose ($P<0.05$). While, Table 2 showed that, serotonin and dopamine in brain tissues were significantly decreased in groups with low and high doses of tramadol when compared to the normal group ($p<0.001$). Also, there was a significant decrease in the level of serotonin and dopamine in the group of the high dose of tramadol when compared to the group of the low dose ($P<0.05$).

Histological studies

Light microscope examination of hematoxylin and eosin (H&E) stained sections of the brain cerebellum of the Normal Control (NC) group rats revealed that the brain tissues were normal with no shrunken cells. On the other hand, the brain tissues stained with H&E stain in the low dose tramadol rats group revealed that, there was few purkinje cells and the cells of granular layer were widely separated. Furthermore, the brain tissues stained with H&E stain in the high dose tramadol rats group showed that, there was a shrunken purkinje cell, some cells had pykinotic nuclei and majority of the cells of granular layer were non-nucleated, few of them had pykinotic nuclei.

Discussion

Tramadol is a pain relief, acting as a central opiate agonist and Central Nervous System (CNS). The chronic use of tramadol can lead to addiction but at a lower risk as compared with other opiates. Most common side effects include fatigue, sedation, and dry mouth [3].

Furthermore, overdose of tramadol may lead to CNS depression, nausea, vomiting, tachycardia, and seizure. First pass metabolism within the liver by demethylation and conjugation reactions is the pharmacokinetics of tramadol. About 90% of tramadol and its metabolites were excreted through the kidneys; the remaining 10% were excreted through the faeces [27].

The present study predicts, the body weights were lost accompanied by decreasing both testosterone level and sperm count in addition to loss of sperm vitality [28]. Where this result in agreement with previous studies, which reported weight loss, decrease of testosterone due to facilitating the damage of spermatogenic cells via increase of reactive species. The deficiency of testosterone would add in producing immature sperm [29].

Reduced plasma levels of luteinizing hormone, follicle stimulating hormone, testosterone, total cholesterol and elevated prolactin and estradiol levels are due to tramadol administration. So, tramadol induced the decrease of sperm count, motility, and numbers of primary spermatocytes, rounded spermatid and leydig cells [30]. Long-term administrations of tramadol have adverse effects on sperm quality and testicular tissues and these effects were dose dependent. Also the negative effects of tramadol on testes are reversible. Also, Heba confirmed that the long-term administration of tramadol reduced the fertility of both male and females.

Opioids can cause serotonin release decrease by inhibiting GABA-ergic neurons. Tramadol binds with low affinity to opioid receptors, resulting in the activation of the descending inhibitory system [31].

Our study favours the hypothesis as Superoxide Dismutase (SOD) and reduced Glutathione (GSH) decline in tramadol treated rats can lead to enhanced H_2O_2 production, stimulation of lipid peroxidation, nitric oxide and protein oxidation. Heba; Ahmed and Kurkar reported that administration of tramadol would increase nitric oxide level, lipid peroxidation and decreased the antioxidant enzymes activities.

Administration of tramadol had abnormalities on both cerebral cortex and testicular tissues associated with oxidative stress in these organs. Also, there was increased apoptosis in both organs which can regresses with withdrawal [32].

The cause of Alzheimer's disease is believed to be genetic with many genes usually involved. Other risk factors include a history of head injuries, depression, or hypertension. The disease process is associated with brain plaques and tangles [33].

Cetin and Dincer revealed that the neurotoxicity and the pathogenesis of Alzheimer's disease (AD) are due to the oxidative stress of β-amyloid peptide (Aβ) [34]. Progressive cognitive dysfunction is due to the neurodegenerative disorder for CNS of AD. The hallmark of AD is the excessive accumulation of neurotoxic Aβ in the brain and the neuropathological lesions in the brain [13].

Conclusion

The results of the present study showed that long term administration of tramadol led to oxidative stress elevation which was the cause of neurodegenerative disorders as Alzheimer's disease and male infertility by the testosterone levels decline.

Statistical analysis

Statistical analysis and correlations were performed using SPSS program version 16. Data are presented as Mean ± standard error mean (SEM). Student "t" test and analysis of variance (ANOVA) followed by Bonferroni's post hoc analysis were used for comparisons between groups. The level of statistical significance was set at probability $P<0.05$.

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