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Original article

Carvacrol Ameliorating Effects on Trimethyltin Chloride-Induced Neurotoxicity by Modulating the Interplay between Nrf2/Keap1/ARE Pathway and Sirt1

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Abstract

Background and objectives: Trimethyltin chloride (TMT) is a chemical with neurotoxic effects on central nervous system. Carvacrol is a phenolic monoterpenoid with antioxidative properties derived from oregano, thyme, and other plants. We aimed to explore carvacrol effects on TMT-induced oxidative damage focusing on nuclear factor erythroid 2-related factor 2 (Nrf2)/ kelch-like ECH associated protein 1 (Keap1)/antioxidant response element (ARE) pathway and Sirt1. **Methods:** Thirty- two male rats were divided into four equal groups. Groups 1 and 2 received normal saline (control) and Dimethyl sulfoxide (DMSO, sham) for 21 days, respectively. Groups 3 and 4 were first treated with TMT (8 mg/kg) and then received normal saline and carvacrol (40 mg/kg) for 21 days, respectively. Finally, the levels of malondialdehyde (MDA), total antioxidant capacity (TAC), and total oxidant status (TOS) in serums and expressions of Nrf2, heme oxygenase-1 (Ho-1), Keap1, NADPH quinone oxidoreductase (NQO-1) and Sirtuin1 (Sirt1) in the hippocampus of the rats were quantified. **Results:** TMT significantly decreased Nrf2, HO1, NQO1, Sirt1 expressions and TAC level, while markedly increased expression of Keap-1 and levels MDA and TOS compared with control groups. Carvacrol treatment significantly upregulated Nrf2, HO1, NQO1, and Sirt1 along with an increase in TAC level as compared with TMT-treated rats. On the other hand, carvacrol caused a significant decrease in the expression of Keap-1 and levels of MDA and TOS compared with controls. **Conclusion:** Our results suggested the potential neuroprotective effects of carvacrol on TMT-triggered neurotoxicity probably by reciprocal regulation of Keap1/Nrf2/ARE pathway and Sirt1 activity.

Keywords: carvacrol; Keap1; Nrf2; Sirt1; trimethyltin chloride

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Introduction

Neurodegeneration is an intricate phenomenon in which neuronal functions and architecture are progressively changed. A plethora of evidence suggests that oxidative stress plays a critical role in neurodegenerative complications [1,2]. Hippocampus is one of the most sensitive regions

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of the central nervous system (CNS) to oxidative stress, which induces defective hippocampal-associated functions of learning, memory, and spatial information processing [3-5]. Our body is generally exposed to both intrinsic and extrinsic sources of free radicals such as inflammatory processes, ischemia/reperfusion injury, X-rays, air pollutants, and industrial chemicals [6,7]. Trimethyltin chloride (TMT) is an industrial chemical that is used in polyvinyl chloride and silicone materials to produce fungicides, plastics, food packages, and kitchen utensils. TMT is also found in domestic water supplies, drinking water, marine habitats, and has been repeatedly employed as a toxic chemical to investigate CNS toxicity [8]. Human exposure to TMT may cause neurological disorders including disorientation, memory impairment, vigilance loss, seizures, and headaches. This compound also causes hippocampal malformations and prevents the development of the brain in animal models [9]. TMT has been reported to increase reactive oxygen species (ROS) formation and induces apoptosis by downregulating antioxidative enzyme-related genes and upregulating apoptosis-inducing genes in the eyes of some animal models [10]. To overcome oxidative stress, the body uses various antioxidative systems. These protective systems involve enzymatic or non-enzymatic antioxidant mechanisms. A primary regulatory mechanism for maintaining cellular redox status is achieved at the transcriptional level, which mainly occurs through the modulation of the nuclear factor erythroid 2-related factor 2/Kelch-like ECH associated protein 1/antioxidant response element (Nrf2/Keap1/ARE) pathway. This pathway controls the expression of more than 100 genes, which their products are involved in the regulation of various oxidative stress-related events and cell survival [11]. Under normal conditions, Keap1 binds Nrf2 and prepares it for proteasomal degradation, but by the accumulation of environmental stressors, Keap1 dissociates from Nrf2 leading to heterodimerization of this protein with Maf family of transcription factors. This causes Nrf2 heterodimer to translocate into the nucleus and bind to the ARE, activating the transcription of many genes including glutathione S-transferase A2 (GSTA2), NADPH quinone oxidoreductase (NQO-1), superoxide dismutase (SOD1), and heme oxygenase-1 (Ho-1) [12]. Sirtuin 1 (Sirt1) has been described to activate Keap1/Nrf2/ARE pathway through decreasing expression of Keap1, enhancing the ability of Nrf2 in binding ARE, and activating transcription of downstream genes [13]. Accumulating data from animal studies to clinical trials suggest that dietary flavonoids protect the neuronal injury and improve memory, learning and cognitive function [14,15]. Carvacrol, as a phenolic monoterpenoid present in some plants such as oregano, thyme, pepperwort, wild bergamot, has shown many antioxidative capacities [16]. This natural compound ameliorates hippocampal neuronal dysfunction and reduces oxidative stress and apoptosis of these neurons in some animal models by various mechanisms [17,18]. However, no previous investigation has focused on molecular mechanistic exploration of the potential protective effect of carvacrol against TMT induced hippocampal neuronal impairment in rat. In the present study, we aimed to examine the neuroprotective effects of carvacrol on expression levels of antioxidant genes including Nrf2, Keap-1, Sirt1, Ho-1and NQO1 in rat hippocampus treated by TMT. Furthermore, we also measured malondialdehyde (MDA), as a product of lipid peroxidation, total antioxidant capacity (TAC), and total oxidant status (TOS) as oxidative stress markers.

Materials and Methods

Ethical consideration

All animal experiments were approved by the Ethics Committee of Arak University of Medical Sciences (IR.ARAKMU.REC.1398.320). All procedures used in the present study were in strict accordance with NIH guidelines for animal care and use.

Chemicals

TMT and carvacrol were purchased from Sigma Company (USA). RNX-plus reagent was obtained from Sinaclon Company (Iran). Complementary DNA synthesis kit and RT-PCR SYBR green I master mix were purchased from Yekta Tajhiz Azma Co. (Iran). Other chemicals used in this work including thiobarbituric acid (TBA), ferric chloride (FeCl3, $6H_2O$), sorbitol sodium, trichloroacetic acid (TCA), 2,4,6-Tripyridyl-s-triazine, 1,1,3,3-tetraethoxy propane (TPTZ) and xylenol orange were purchased from Sigma Aldrich (Germany).

Animals

A total of 32 adult male Wistar rats weighing 180-220 g were provided by the animal breeding center of Arak University of Medical Sciences (Arak, Iran). All rats were housed in a temperature-controlled room (22 ± 2 °C). The rats were maintained on a 12-h light/dark schedule and fed ad libitum.

Experimental protocol and groups

TMT was purchased from Sigma–Aldrich (St. Louis, USA). To prepare the carvacrol solution (Sigma, St. Louis, USA) was uniformly dispersed in normal saline containing 25 % DMSO. Rats were randomly divided into four groups (n=8 each). Groups 1 and 2 (as control and sham groups) were injected intraperitoneally (i.p.) with normal saline and DMSO (as the vehicle of carvacrol), for 21 days respectively. Groups 3, 4, were initially treated with a single dose of TMT (8 mg/kg, i.p.) [19]. After 24 h, group 3 received normal saline and group 4 was administered carvacrol (40 mg/kg, i.p.) for 21 days [20,21]. At the end of the experimental period, all animals were anesthetized with ketamine (50 mg/kg, i.p.)/ xylazine (10 mg/kg, i.p.) and their blood samples were taken directly from the heart and centrifuged at 2500 rpm for 10 min. Brain tissues of the rats were removed and then the hippocampal regions were separated according to the atlas of Paxinos and Watson. All tissues were immediately frozen with liquid nitrogen. Serum samples and hippocampal tissues were stored at –70 °C until further analysis.

Oxidative stress markers measurement

Oxidant and antioxidant markers were determined in serum samples using biochemical methods. The level of MDA, as a biomarker for lipid peroxidation, was measured by the thiobarbituric acid (TBA) method described by Satho [22]. First, serums were mixed with 20% trichloroacetic acid to prepare a precipitate. Then, 0.05 M H_2SO_4 and TBA (0.2% in 2 M sodium sulfate) were added to the precipitate and heated for 30 min in boiling water. Finally, lipid peroxidation end products were extracted using n-butanol and absorbance was measured at 532 nm.

Total antioxidant capacity (TAC) was assayed based on the method of Benzie, et al. [23]. This method uses the ferric reducing ability of plasma (Fe^{3+} to Fe^{2+}). By adding tripyridyltriazine (TPTZ) to samples, a blue color complex was created between Fe^{2+} and TPTZ and its absorbance was measured at 593 nm.

The level of total oxidant status (TOS) was measured using the Erel method [24]. Briefly, serum oxidants in the sample oxidized Fe^{2+} to Fe^{3+} by mixing serums with FOX1 reagent (20 mM H_2SO_4, 200 μM xylenol orange, 150 mM sorbitol and 250 μM Fe^{2+} at pH was 1.7). Then, the xylenol orange formed a colored complex with ferric ions and absorbance of the complex was measured at 560 nm.

RNA Extraction and cDNA synthesis

Total RNA was extracted from hippocampal tissues using RNX-plus reagent according to the manufacturer's instruction. The extracted RNA specimens were then quantified by a Nanodrop spectrophotometer and also the purity of extracted RNAs was estimated. A260/A280 ratios of 1.8-2 were considered high purity RNA. Two μg, total RNA was reverse transcribed into cDNA in a total volume of 20 μL by using cDNA Synthesis Kit. Finally, the synthesized cDNAs were stored at −70 °C until further experiments.

Real-Time qRT-PCR

Real-Time -PCR was performed with a SYBR green master mix) using a light cycler Real-time-PCR system. Amplification conditions were 40 cycles for 5 min at 95 °C, 20 s at 56 °C, and 10 s at 72 °C. The primer sequences specific to HO-1, Keap-1, NQO1, Sirt1, and Nrf2 primer sequences were designed using Allele ID software. To normalize the expression levels of the measured genes, cyclophilin A (cyclo A) gene was used as an internal control. The forward and reverse primer sequences for evaluating the mentioned genes are shown in Table 1.

Table 1. Primer sequences used in this study

Gene	Forward primer	Reverse primer
HO-1	5′- CTAGCCTGGTTCAAGATACTAC -3′	5′- GGAAACTGAGTGTGAGGAC -3′
Keap-1	5′- CAGCGTGGAGAGATATGAG -3′	5′- AGTAACATTCTGCCGAGTT -3′
NQO1	5′- GTCATCTCTGGCGTATAAGG -3′	5′- CAATGGGAACTGAAATATCACC -3′
Sirt 1	5′- CATCTTGCCTGATTTGTAAA -3′	5′- AACTTCATCTTTGTCATACTTC -3′
Nrf2	5′- ACAACTGGATGAAGAGACCG -3′	5′- TGTGGGCAACCTGGGAGTAG -3′
Cyclo A	5′- GGCAAATGCTGGACCAAACAC -3′	5′-TTAGAGTTGTCCACAGTCGGAGATG-3′

The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to calculate relative changes in the expression levels (fold changes) of studied genes in the hippocampal tissues in comparison to control tissues.

Statistical analysis

Results are shown as mean±SEM. One-way ANOVA with Tukey or LSD post hoc test was used to determine the difference in the experimental variables between the studied groups. A p-value of<0.05 was considered statistically significant. Data were analyzed with SPSS16 analytic software (SPSS, Inc., USA).

Results and Discussion

Figure 1A and 1B indicate that treatment of rats with TMT that caused a significant increase in the concentration of MDA and TOS in comparison to control (normal saline) and sham (DMSO) groups (p=0.001). MDA and TOS levels were significantly decreased following treatment of the rats with carvacrol as compared with the TMT-treated rats (p=0.01). This treatment led to the returning of MDA and TOS levels back to the control and sham levels. On the other hand, treatment of rats with TMT considerably reduced the TAC levels when compared with control and sham groups as depicted in figure 1C (p=0.001). Inversely, carvacrol exposure, significantly elevated TAC compared to TMT group (p=0.001), and TAC level returned to those of saline or DMSO-treated animals.

Figure 2A illustrates that treatment of rats with TMT resulted in a significant reduction in the expression levels of Nrf2 in the hippocampus of the animals in comparison to control and sham groups (p=0.01). Nevertheless, carvacrol treatment considerably augmented Nrf2 mRNA expression when compared with TMT-treated group (p=0.003). As apparent in this figure, this treatment caused the enhancement of Nrf2 expression to the levels equal to control and sham groups.

Figure 2B shows that TMT treatment caused a significant elevation in the expression levels of Keap-1 in the hippocampus tissues of the rats in comparison to control and sham groups (p=0.01). However, carvacrol treatment considerably reduced Keap-1 expression levels as compared with TMT-treated group (p=0.02), and Keap-1 expression levels returned to those of saline or DMSO- treated animals.

As depicted in figure 3A, treating the rats with TMT led to a significant decline in the expression of HO1 mRNA in the hippocampus of these animals compared with control and sham groups (p=0.001, p=0.000). Carvacrol treatment markedly enhanced the expression HO1 compared with TMT-treated group (p=0.009). HO-1 expression levels returned to those of saline or DMSO treated animals.

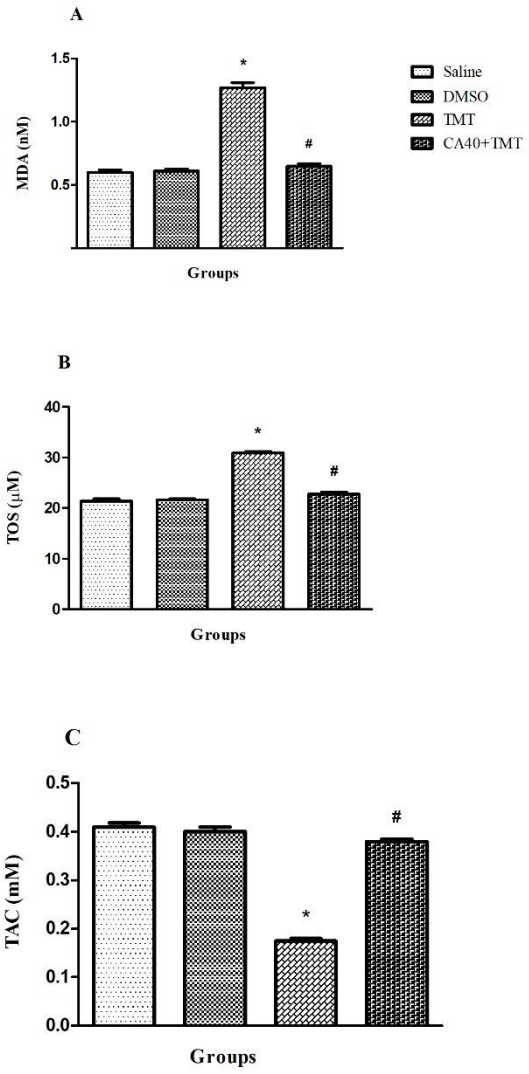

Figure 1. Effects of carvacrol on malondialdehyde (MDA) (A), total oxidant status (TOS) (B), and total antioxidant capacity (TAC) (C) serum levels in TMT treated rats; values are expressed as the mean ± SEM (n = 8 per group). CA: carvacrol; TMT: Trimethyltin chloride; *p<0.05 as compared with control and sham groups (saline and DMSO); #p<0.05 as compared with TMT group

Figure 3B shows the changes in the expression levels of NQO1 mRNA in hippocampus tissues

of rats following treatment agents. As demonstrated in this figure, treating the animals with TMT induced a significant decrease in the expression of NQO1 mRNA compared to the control and sham groups (p=0.001, p=000). Carvacrol treatment considerably increased NQO1 expression levels as compared with TMT-treated group (p=0.01). However, carvacrol treatment failed to return levels of NQO1 mRNA to those of saline- treated animals.

Figure 3C illustrates that exposure of rats with TMT remarkably inhibited the hippocampus SIRT1 expression in our tested animals in comparison to control and sham groups (p=0.001, p=0.000). Carvacrol treatment significantly incremented the SIRT1 mRNA expression compared to TMT-treated group (p=0.006). However, this treatment could not increase SIRT1 expression to the levels similar to control and sham groups.

TMT has been reported to have an association with neurodegenerative diseases via induction of oxidative stress. Carvacrol is a monoterpenoid that has strong antioxidant properties. For this reason, the present study aimed to look into the antioxidant potential of carvacrol against TMT neurotoxicity by focusing on Nrf2/ARE/ Keap-1 pathway.

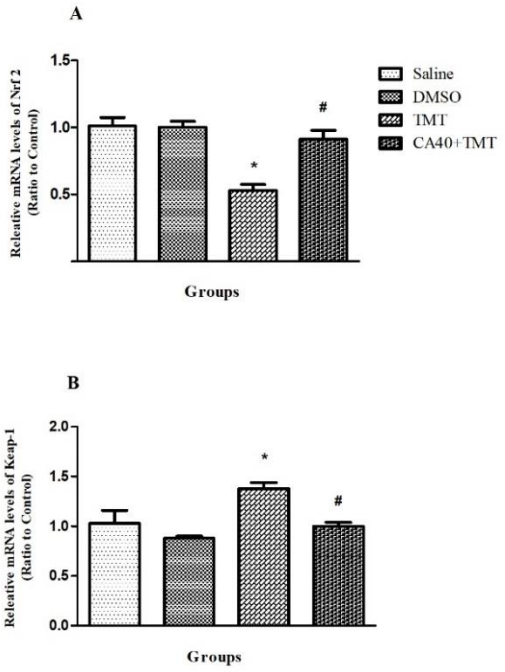

Figure 2. Effects of carvacrol on Nrf2 (A) and Keap-1(B) gene expression of the hippocampus in TMT treated rats; values are expressed as the mean ± SEM (n = 8 per group); CA: carvacrol; TMT: Trimethyltin chloride; *p<0.05 compared with control and sham groups (saline and DMSO); #p<0.05 compared with TMT group

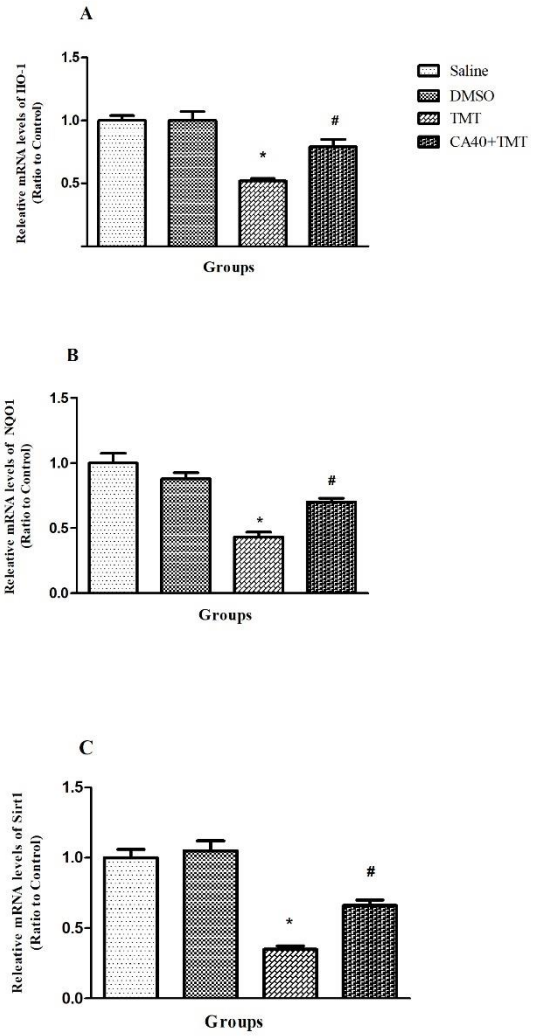

Figure 3. Effects of carvacrol on HO1 (A), NQO1 (B), and Sirt1(C) gene expression of the hippocampus in TMT treated rat; values are expressed as the mean ± SEM (n=8 per group); CA: carvacrol; TMT: Trimethyltin chloride ;*p<0.05 as compared with control and sham groups (saline and DMSO); #p<0.05 as compared with TMT group

Our data showed that TMT treatment of rats significantly elevated the concentration of MDA and TOS, but decreased TAC in serum samples of these animals in comparison to control and sham groups. However, carvacrol at the dose of 40 mg/kg inversed these effects by decreasing MDA and TOS and increasing TAC in animal serums compared to TMT-treated groups.

We also observed that TMT treatment markedly enhanced the expression levels of Keap1 and considerably inhibited the expression of Nrf2, Ho1, NQO1, and Sirt1 mRNAs in hippocampus tissues of the rats as compared with control and sham groups. On the other hand, our experiments

provided evidence to uncover that carvacrol significantly amplified expressions of Nrf2, Ho1, NQO1, and Sirt1 genes and lowered the level of Keap1 expression in hippocampus tissues of the treated rats compared with corresponding TMT-treated animals.

Many previous studies, which were conducted to investigate TMT or its derivatives effects on oxidative stress induction in various organs have acquired data that are in line with our results. In a study, TMT has been described to cause hippocampus intoxication along with cognitive deficit in mice models. These detrimental effects were ameliorated with coenzyme Q10 as an antioxidant against oxidative stress-induced neuronal necrosis [25]. This antioxidant coenzyme has been shown to act by modulating Sirt1/Nrf2 axis [26]. Yoneyama, et al. showed the enhancement of MDA in the hippocampus of mice following TMT treatment. Conclusively, they indicated that one possible mechanism for TMT-induced neuronal injury may be due to oxidative stress-induced activation of apoptosis signals [27]. Liang, et al. attributed the antiproliferatory and apoptosis-inducing activities of TMT to oxidative stress and Nuclear Factor Kappa B (NF-κB) signaling pathway activation [28]. However, Zhao, et al. ascribed apoptosis inducing capacity of TMT to inhibitory effects of this compound phosphorylated glycogen synthase kinase-3β (GSK-3β), Sonic Hedgehog, and PI3K/Akt pathways. They also detected TMT induced oxidative stress in mouse neural crest-derived cells, which was confirmed by increased generation of ROS and MDA and decreased activity of SOD [29]. Also, TMT seems to involve some other pathogenic mechanisms to induce neurodegeneration, which may include neuroinflammation, high intracellular calcium, and oxidative stress [30,31]. In our present investigation, TMT augmented Keap1 expression, but decreased Nrf2, HO1, NQO1, and Sirt1 mRNAs, suggesting that Nrf2 has been sequestered by Keap1, degraded, and didn't upregulate its target genes. As described before, Sirt1 affects the Keap1/Nrf2/ARE pathway by blocking Keap1 and activating the transcriptional ability of Nrf2 [13]. In the current study, TMT probably had preventive effects on the Keap1/Nrf2/ARE pathway by downregulating Sirt1 expression. Our data also illustrated the counter regulating effects of carvacrol on TMT-induced oxidative stress. These protective effects of carvacrol were converged on decreased oxidative markers and increased antioxidant status. This compound by blocking Keap1 caused incrementation of Nrf2, HO1, and NQO1 expressions, indicating that Nrf2 has been released from the physical tethering of Keap1 and translocated into nucleus enhancing the expression of its downstream genes such as HO1 and NQO1. In parallel, carvacrol markedly induced Sirt1, which might assist in activating the function of Keap1/Nrf2/ARE pathway. Neuroprotective effects of carvacrol have been numerously reported in previous investigations. Samarghandian, et al. showed that carvacrol significantly reduced the concentration of MDA and induced the activities of CAT, SOD, and some other antioxidant enzymes in rat's brain. This natural product also alleviated oxidative stress overload in liver and kidney tissues of the rats [18]. Carvacrol can ameliorate cyclophosphamide-triggered oxidative stress in rat kidney by decreasing MDA, TOS, and oxidative stress indexes (OSI) levels and increasing GSH, SOD, CAT, and TAC levels in this tissue [32]. Zou, et al, provided evidence to prove the antioxidative activities of oregano essential oil against H_2O_2-induced oxidative stress in intestine-derived cells. They experimentally revealed that oregano essential oil significantly elevated the expression of Nrf2 and its target genes in these cells. Interestingly, they asserted that the antioxidative activities of oregano essential oil are attributable to two main components of oregano essential oil, which include carvacrol and thymol [33].

Conclusion

In the present investigation, carvacrol probably exerted its antioxidative effects against TMT-induced oxidative stress through the modulation of two well-characterized factors that comprise the Keap1/Nrf2/ARE pathway and Sirt1 molecule. These two factors, in turn, played their role by regulating downstream targets. Likewise, Keap1/Nrf2/ARE pathway activation led to the upregulation of antioxidative enzymes and Sirt1 presumably has emerged as the positive regulator of this pathway to overcome oxidative damage. According to our data, carvacrol reciprocally orchestrated these antioxidative pathways and hence can be considered as a promising tool in battling against overwhelming oxidative stress in neuronal systems.

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Author contributions
Farideh Jalali Mashayekhi, Mohammad Hasan Sakhaie were involved in study conception and design; Farzaneh Babak, Sadegh Rajabi, Farideh Jalali Mashayekhi designed and performed experiments; all authors participated in analysis and interpretation of results, reviewed the results and approved the final version of the manuscript.

Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

Ho-1: heme oxygenase-1; GSTA2: glutathione S-transferase A2; Keap-1: Kelch-like ECH

associated protein 1; MDA: malondialdehyde; Nrf2: nuclear factor erythroid 2-related factor 2; NQO1: NADPH quinone oxidoreductase; OSI: oxidative stress indexes; Sirt1: Sirtuin1; SOD: superoxide dismutase; TMT: trimethyltin chloride; TAC: total antioxidant capacity; TBA: thiobarbituric acid; TOS: total oxidant status; TPTZ: tripyridyltriazine; ROS: reactive oxygen species