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Research Paper:

Pentoxifylline Protects Against Hippocampal Damage and Memory Impairment Induced by Trimethyltin

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ABSTRACT

Background: Trimethyltin (TMT) is a toxic agent that causes oxidative stress, a laboratory model for inducing hippocampal injuries. Pentoxifylline (PTX) inhibits phosphodiesterase, inflammation and oxidative stress. This study evaluated the neuroprotective effects of PTX on injuries induced by TMT in the hippocampus.

Methods: Sixty male Wistar rats were divided into five groups of 12 each. Group 1 received normal saline while Group 2 received a single dose of TMT (8 mg/kg). The other four groups received TMT at 8 mg/kg plus 60, 100 or 120 mg/kg PTX twice daily for six consecutive days. The rats' working and reference memory were investigated, using radial arm maze tasks. At the end of the experiments, the rats' brains were removed and processed for histological study of the hippocampus.

Results: The TMT treatment prolonged the four baited arms tasks ($P<0.001$), while the PTX treatment at 60, 100 or 120 mg/kg significantly reduced the effects of TMT on the spatial memory ($P<0.01$). The working and reference memory errors significantly increased in the TMT group compared to the controls ($P<0.001$) while the PTX treatment significantly reduced the TMT effect ($P<0.001$). Also, TMT increased the number of pyknotic cells in the hippocampus ($P<0.001$), while PTX significantly decreased the mean number of pyknotic cells ($P<0.05$).

Conclusion: The findings suggest that PTX can protect against the memory deficit and deleterious effects on rat hippocampus induced by TMT neurotoxicity. Thus, PTX is likely to be a potential agent to prevent against the neurotoxicity induced by TMT.

Keywords: Hippocampus, Neurotoxicity, Pyknotic cells, Neurodegeneration, TMT, Spatial memory

Introduction

Neurodegenerative diseases are disabling disorders of the Central Nervous System (CNS). The molecular mechanisms of the disorders may include mitochondrial dysfunction, cell membrane peroxidation, glutamate excitation, calcium overload, and activation of pro-apoptotic proteins [1]. Hippocampus is one of the vital constituents of the brain. It is also believed that some deleterious conditions of the brain, such as trauma, seizure, hypoxia, ischemia, among others, may induce injury to the hippocampus [2-4].

Trimethyltin (TMT) is a fungicidal agent and stabilizer of plastic materials. This agent is used as a convenient compound to induce experimental degenerative model of hippocampus injury in rodents, manifested by cogni-

tive deficits, inflammation, neuronal death and apoptosis [5]. The clinical signs of hippocampal injury in rodent are tremor, convulsion, hyperactivity, tail mutilation, rarring and aggressive behaviors [6]. It has been shown that treating mice with TMT causes neuronal loss in the dentate gyrus of the hippocampus [7]. Although the mechanisms of TMT action in the hippocampus are not completely elucidated, experimental findings suggest that this agent affects mitochondria and membrane proteins, leading to the release of cytochrome C [8]. Other studies have suggested that the mechanisms of neurotoxicity of TMT may be related to impairment of neurotransmitters, glutamate excitotoxicity, formation of Reactive Oxygen Species (ROS), and the elevated expression of Necrotizing Factor (NF-kB) [9, 10]. Moreover, the administration of TMT can activate the microglia and induce neuronal cell death [11]. It may upregulate the expression of inducible Nitric Oxide Synthase (iNOS), which augments the Reactive Nitrogen Species (RNS) and apoptosis in the hippocampus [12]. Exposure to TMT also damages pyramidal cells in dorsal hippocampus and the hillus through astrogliosis and toxicity to the microglia [13]. Further, it has been shown that TMT organotin compound induces spatial learning deficit, based on water maze and radial arm maze tests in rats [14].

Pentoxifylline (PTX) is an alkylxanthine phosphodiesterase inhibitor that reduces the plasma viscosity and is used to treat some vascular disorders. Noyan, et al. reported that PTX has the potential to be used as an immunomodulatory and ROS scavenging agent [15]. The anti-inflammatory effect of PTX is thought to be due to the inhibition of glial cell activities, which are the primary sources of releasing inflammatory cytokines [16]. This agent plays a protective role against experimental neurotoxicity and neuropathic pain [16, 17]. It downregulates the pro-inflammatory Tumor Necrosis Factor-α (TNF-α) and interleukin-1β in neurons, and enhances the anti-inflammatory effects of cytokines [18]. The study by Banfi et al. revealed that PTX reduced brain damage dose dependently by inhibiting the inflammatory events [19]. Another study showed that using PTX in an experimental model of status epilepticus improved behavioral learning, cognition, alleviated seizures, and reduced neuronal death by inhibiting the oxidative stress, and augmented monoamines in the hippocampus [20]. Another in vitro study has revealed that PTX plays a major anti-inflammatory and anti-apoptotic role by inhibiting TNF-α and the caspase-dependent pathways [21].

The aim of this study was to investigate the neuroprotective effects of PTX on neuronal injuries, and deficits in the behavior, learning and memory that are induced by TMT in rat hippocampus.

Materials and Methods

Animals: Sixty male Wistar rats, weighing 190-220 g, were randomly divided into five groups of 12 each. They were housed under controlled conditions of temperature (21-23°C), humidity (50%-60%), lighting (12 h light–dark cycle), and free access to food and water. The established animal care procedures were observed to reduce stress in rats, as approved by the Review Board and Ethics Committee of Arak University of Medical Sciences (Registration #: IR.ARAKMU.REC 1394.318). The five experimental groups (n=12 each) were as follows:

- Control Group was injected with normal saline intraperitoneally (IP).

- TMT Group received a single dose of TMT at 8 mg/kg (Sigma, St. Louis, USA), which was dissolved in normal saline and injected IP on the first day of the study.

- TMT+PTX60 Group received 60 mg/kg PTX.

- TMT+PTX100 Group received 100 mg/kg PTX.

- TMT+PTX120 Group received 120 mg/kg PTX.

PTX: The PTX compound was obtained from Abcam Co. (Cambridge, UK), dissolved in normal saline and administrated IP twice daily, starting 24 hours after the TMT injection over six consecutive days for a total of 12 PTX injections during the study.

Radial Arm Maze (RAM): The RAM apparatus consisted of eight arms (50×15×15cm), marked as number 1 to 8, were made of black plexiglass and were radiating outward from a central point. The arms were separated from each other by a sliding gate.

During the trials, additional visual cues were placed at similar points in the experimental space. Twenty-four hours after the last PTX administration, the RAM tasks were performed for eight consecutive days (9 a.m. to 12 noon). One day before starting the RAM tasks, the rats were placed in the apparatus to become familiar with the experimental environment. To provoke their exploring behavior, the food was removed from the animal cage 2 hr before each trail.

Throughout the training period and for adaptation to the experimental environment, each animal was placed

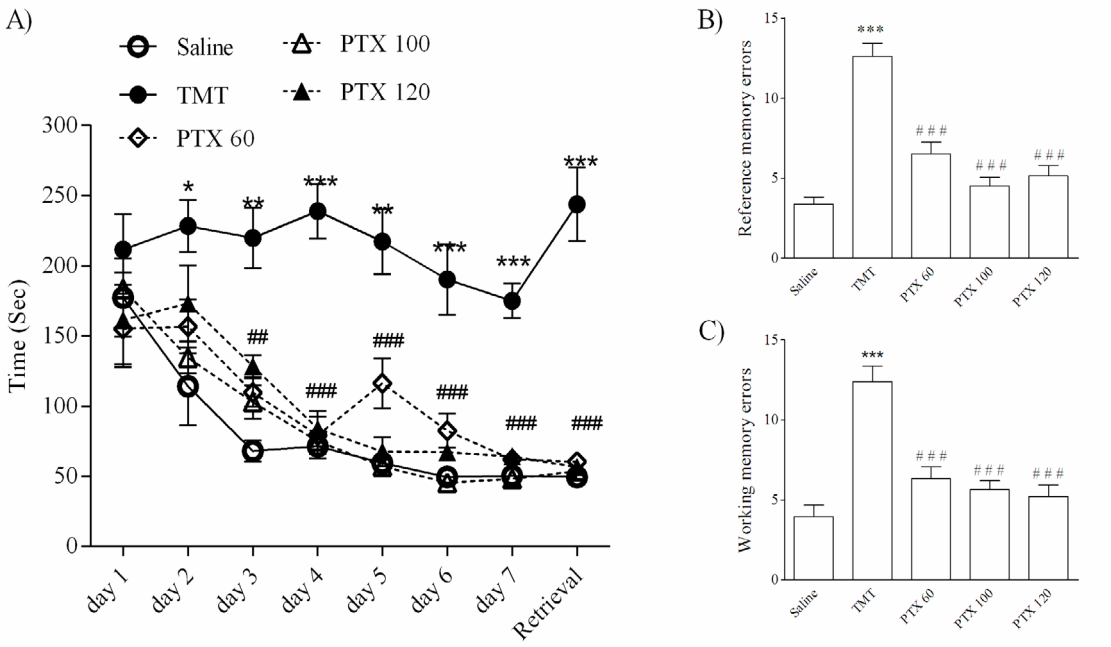

Figure 1. PTX administration ameliorated the TMT-induced memory deficit in rats

Total time to finish the baited arms (A) was significantly increased by TMT. While all of the three doses of PTX significantly reduced this TMT effect. TMT significantly increased the numbers of working and reference memory errors, whereas PTX at all three doses (60, 100, and 120 mg/kg) significantly decreased the effects of TMT on memory errors. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ vs. Control; $^{\#\#\#}P<0.01$ vs TMT.

on the octagonal central platform of the maze for 30 seconds. Then all gates were opened and rats were allowed to freely explore the maze for five minutes. During the RAM tasks, four out of the eight arms contained the same bait for the daily training. The baiting was arranged in the same order throughout the experiment and all rats were trained on this arrangement. The rats had access to all baited arms to explore and eat the foods within the five minutes. The foods were removed from the maze after that period and the arms were cleaned with 40% ethanol between trials. The following variables were used for the analysis according to the established procedures [22]:

1. Latency: total time taken to finish with the baited arms

2. Working Memory Error (WME): re-entry to a previous baited arm where the food was taken

3. Reference Memory Error (RME): entering into an unbaited arm.

Histological study: Prior to the histological analysis, the rats were anesthetized IP with ketamine and xylazine at 60 mg/kg and 10 mg/kg of each drug, respectively. Transcardiac perfusion was performed with 0.9% normal saline, followed by 4% buffered paraformaldehyde fixative in 0.1 M phosphate buffered saline at pH 7.4. The brains were carefully removed from the rat skulls and immersed in the fixative solution overnight. After the tissues were dehydrated in ethanol and cleared with xylene, they were embeded in paraffin and coronal sections were made of hippocampus at 5µm thickness (2.28 to 3.82 mm post bregma) [23]. Sections were then dehydrated serially in increasing alcohol concentrations, stained with cresyl violet, and mounted. The slides were examined under light microscopy at x40 magnification. The microscopic examinations focused on pyknotic neurons, cells with dark nuclei, loss or unusual clumping of heterochromatin, and images were photographed by an Olympus microscope (BX51, Japan). To determine the number of Cornu Ammonis 1 (CA1) pyknotic neurons, five sections from each sample were randomly selected and the cells in four fields were counted in each optic field.

Statistical analyses: All data were presented as Means±SEM. Total time to finish the baited arms were analyzed by two-way ANOVA repeated measures followed by Bonferroni post-test. Numbers of working and reference memory errors were analyzed by the Kruskal-Wallis test followed by the Dunn multiple comparison tests. One-way ANOVA with Tukey's post-hoc test was used to evaluate the mean number of necrotic neurons in the CA1 area of hippocampus samples.

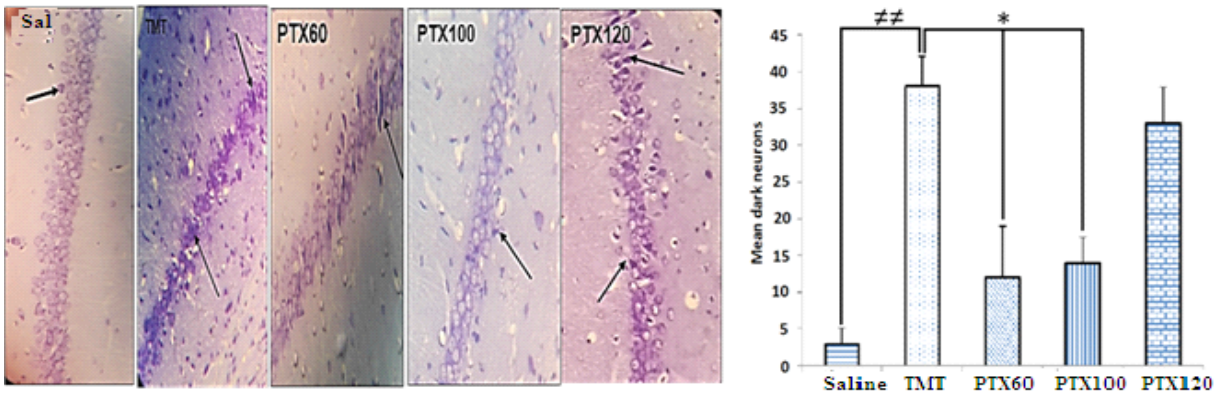

Figure 2. PTX reduced the number of pyknotic neurons produced by TMT

Photomicrograph of Nissl staining in CA1 region of hippocampus of the experimental groups (arrows show pyknotic neurons). Mean number of pyknotic neurons significantly increased after TMT treatment, while PTX (60 & 100 mg/kg) repaired this effect of TMT. Magnification x40, the bar chart shows the rate of pyknotic neurons. N=7 for all groups; *P<0.05 vs Controls, ≠P<0.01 vs TMT treatment.

Results

Spatial memory study: As shown in Figure 1A, TMT significantly increased the delay time taken by the rats to finish with 4 baited arms (P<0.001). The PTX administration at the three given doses significantly reduced the effect of TMT (P<0.001). As demonstrated in Figures 1B and 1C, TMT significantly increased the working and reference memory errors (P<0.001) while PTX significantly decreased the effects of TMT on memory errors at the three administered doses of 60, 100, and 120 mg/kg (P<0.001).

Histological study: As seen in Figure 2, the administration of TMT significantly increased the numbers of pyknotic neurons in the nissl stained CA1 region of the hippocampus (P<0.001) compared to the group treated with normal saline. Further, PTX at 60 and 100 mg/kg significantly decreased the numbers of pyknotic neurons in the CA1 area of the hippocampus (P<0.05) compared to the group treated with TMT alone. However, there were no significant differences noted for the PTX treatment at 120 mg/kg compared to that in the TMT group.

Discussion

This study was conducted to investigate the neuroprotective effects of PTX on the induced neuronal injury by TMT in the rat hippocampus. The results indicated that the chronic administration of PTX significantly reduced histological damages in the CA1 area of the hippocampus and improved the spatial memory impairment caused by TMT. This toxin has been used previously in animal studies to induce neuronal damage in the hippocampus and produce impairment in spatial learning and memory [5, 6, 24]. The findings of the current study confirmed the neuronal damage based on the histological examinations following TMT administration to the rats. These histological findings were also associated with spatial memory impairment detected on the radial maze tasks. It is believed that TMT induces oxidative stress, inflammation, and neuronal death in the hippocampus through the stimulation of microglia and astrocytes. These events are associated with the release of pro-inflammatory cytokines and stress related factors, which promote inflammation in neurons [5, 6, 9].

Neuro-inflammatory conditions are known as the major contributors to the development of neurodegenerative and cognitive disorders [25]. Initially, the inflammation leads to neuronal death mediated by cytokines, such as interleukins 1α, β and 6, TNF-α and the activation of iNOS [26, 27]. Earlier findings have also shown TMT-induced apoptosis in an immortalized hippocampal cell line (HT-22). This is believed to be due to the excessive ROS/RNS generation secondary to the up-regulation of NF-κB, iNOS and Bax expression [12]. Studies have demonstrated that the number of TUNEL-positive pyramidal neurons in the CA1 and CA3 regions of hippocampus increased to a maximal level after 5 days of exposure to TMT while the number of Nissl-positive mature neurons decreased 21 days after the TMT treatment [27, 28]. The findings from these studies are consistent with the histological results of the current study regarding the neuronal damages found in the CA1 region of the rats' hippocampus.

With respect to the important role of hippocampus in learning and memory, the impaired learning documented in the rats following TMT treatments, could be linked to

neuronal damages in the CA1 region of the hippocampus. As mentioned earlier, the TMT toxicity might have increased the oxidative stress and mitochondrial dysfunction in the hippocampus, likely to be associated with gliosis and spatial learning impairment in rats [29]. The mechanisms by which oxidative stress can impair cognitive behavior may be related to alteration in the Brain-Derived Neurotrophic Factor (BDNF). This factor is produced by hippocampus neurons and has a critical role in synaptic plasticity, neuronal excitability, and learning and memory tasks. The rise in the oxidative stressors in the CNS can decrease the BDNF expression while increasing the lipid peroxidation, both of which can lead to impairment in cognitive functions, learning memory and neuroplasticity [30, 31].

The findings of the current study suggest that PTX at all of the three doses (60, 100, and 120 mg/kg) might have protected the hippocampus against the adverse effects of TMT on the spatial learning and memory functions. Also, our histological results were consistent with the findings of previous studies that have shown PTX treatment to have a discernible attenuation of cell loss in the CA1, CA3 and hilus areas of hippocampus [20]. Further, PTX may modulate the inflammatory response by down-regulating the production of various pro- and anti-inflammatory cytokines. In previous in vivo studies, PTX at 200 mg/kg diminished the spatial memory deficit, which was probably mediated by the inhibition of TNFα synthesis [32, 33]. Also, in a rat model of neuropathic pain, it was reported that PTX dose-dependently reduced the activity of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, and enhanced the IL-10 activity [16]. It has also been reported that PTX alleviates the memory impairment secondary to hypoxia or ischemia by reducing the ratio of BAX/Bcl-2 protein levels [17, 24]. Previous studies have also shown that the suppression of apoptosis via controlling the Bax/Bcl-2 ratio in the hippocampus has improved the learning and memory performance in animal models [32, 33]. Finally, Tariq et al. have reported that PTX alleviated the neuronal cell loss and sprouting of mossy fibers in the hippocampal tissue damage induced by lithium-pilocarpine. These findings suggest the oxidative stress reduction, and reversal of the depletion of dopamine and 5-hydroxytryptamine in the hippocampus by PTX treatment [20].

Conclusions

The current study findings justify the neuroprotective effects of PTX against the spatial memory deficit and histological damage in the hippocampus induced by TMT neurotoxicity. These PTX effects, as supported by histological findings, suggest a decline in the neuronal necrosis through modulation of the anti-inflammatory processes and improved neuro-protective mechanisms.

Limitations of the study: This study had limited laboratory resources to assess a variety of anti-inflammatory mediators at molecular levels.

Recommendations for future research: The role of anti-inflammatory cytokines should be considered in the future studies.

Ethical Considerations

Compliance with ethical guidelines

All ethical principles were observed in conducting this research, including the principles governing the use of animals in experimental studies as approved by the Ethics Committee of Scientific Research at Arak University of Medical

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Author's contributions

Conceptualization and Methodology: Sakhaie MH, Mohammad-Hosseini, and Sadegh M; Data Analyses and Experiments: Sadegh M and Sakhaie MH; Writing – original draft and Writing, review, and editing: Babaei S, Sakhaie MH and Sadegh M.

Conflict of interest

The authors declared no conflict of interests.

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