International Journal of Medical Laboratory 2016;3(1):43-49.

Original Article

Effects of Thymol on Serum Biochemical and Antioxidant Indices in Kindled Rats

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Article history
Received 15 Nov 2015
Accepted 17 Feb 2016
Available online 11 Mar 2016

Key words
Epilepsy
IL-1β
Malondialdehyde
Thymol
TNF-α

A B S T R A C T

Background and Aims: Kindling is regarded as an experimental model of temporal lobe epilepsy that reflects a process of progressive and persistent intensification of seizure. Experimental and clinical evidence suggest that there is an interrelationship among epileptogenesis, local inflammation and antioxidant activity. In this study, the possible antioxidant and anti-inflammatory effects of thymol was investigated in epileptic rats.

Materials and Methods: Kindling was induced by repeated injections of intraperitoneal pentylenetetrazole (PTZ) every other day. Then, the rat hippocampi were isolated, weighed and prepared as a 5% tissue homogenate in ice-cold 0.9% saline solution. Serum malondialdehyde (MDA) and superoxide dismutase (SOD) levels were assessed by thiobarbituric acid reacting substance and nitroblue tetrazolium methods respectively. Hyppocampal tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) were evaluated using enzyme-linked immunosorbent assay method.

Results: As the study findings revealed, epileptic seizures increase the serum level of MDA, hyppocampal levels of TNF-α as well as IL-1β and decrease the SOD activity.

Conclusions: thymol exerts anticonvulsant activity through the antioxidant and anti-inflammatory mechanisms.

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Introduction

Epilepsy can be mentioned as one of the most frequent disorders of central nervous system, being suffered approximately by one individual out of 100 individuals of the world's population. Recurrent spontaneously abnormal electrical discharge of restricted neurons in the central nervous system leads to epilepsy in patients. In spite of the development of effective antiepileptic drugs for epilepsy treatment, drug resistance and their side effects specially in high doses are the main causes of exploring the new strategies and methods for the medical treatment of epileptic patients [1]. Natural compounds that prevent cellular oxidation, retard the mitochondrial dysfunction and have anti-inflammatory useful effects on the r treatment of a wide spectrum of diseases. Thymol and carvacrol are regarded as natural isomeric monoterpene phenolic compounds widely used as a remedy in Iranian folklore medicine having some health benefits. In addition to their antimicrobial properties, both compounds also indicate a potent anti-inflammatory and antioxidant properties that can be considered as the underlying mechanism of their anxiolytic [2] and anticonvulsant [3] effects. In contrast with carvacrol, antioxidant effects of thymol have been demonstrated in the kindled rats [4]. Serum oxidant/antioxidant balance was changed in the most abnormalities such as the epileptic diseases [5]. Oxidative stress results in cell death and alterations in cell functions such as membrane permeability and excitability through the lipids, as well as protein and enzyme oxidation [6]. Similarly, oxidative stress leads to mitochondrial dysfunctions and, in turn, mitochondrial oxidative stress contributes to epilepsy [7]. This mitochondrial dysfunction can affect reactive oxygen species production which can result in self injury, macromolecular function, bioenergetics, glutamate excitotoxicity, neuronal death and neuronal excitability as well as increased seizure susceptibility [8, 9].

Suppression of brain inflammation has been reported to be a mechanism of antiepileptic drug or potentiate their effects. However, beneficial effects of some anti-inflammatory drugs such as adrenocorticotropin, celecoxib and intravenous immunoglobulin (IVIG) have been reported in some epileptic diseases. Inflammatory cytokines including interleukin-1β (IL-1 β) and tumor necrosis factor-α (TNF-α) are mainly released by astrocytes and microglia after the seizure [10]. Consequently, the over-production of the inflammatory mediators can lead to modulation of neuronal ion currents, increased synaptic glutamate release, and decreased gamma-aminobutyric acid neurotransmission. Taken together, the inflammatory mediators lead to hyperexcitability in the central nervous system through the aforementioned mechanisms. Therefore, natural compounds with antioxidant and anti-inflammatory properties, targeting the oxidative stress and inflammatory response, may be a beneficial complementary medicine in order to manage the epilepsy.

The present study was conducted to clarify the antiepileptic effects and possible neuroprotective

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mechanisms of thymol against pentylenetetrazol (PTZ)-induced kindling in rats.

Materials and Methods

Animals

The present study was conducted on the male Wistar rats (200-220 gr)maintained at 25± 2ºC, dark/light cycle and 40–50% of humidity. The animals had access to food and water ad libitum and they had all the equal or same condition. Experiments were carried out after a week of acclimatization according to the guidelines of Animal Ethics Committee of the Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Drugs and treatment schedule

The rats were allocated into five groups, consisting of seven animals in each group. PTZ was purchased from Sigma-Aldrich (UK) and dissolved in 0.9% sterile saline on a weight/volume basis on the day of use. Thymol (Sigma-Aldrich, UK) was first mixed with 100 µl of Tween 80 (20% w/v) before making up the volume with the distilled water and was then administered intraperitoneally at different doses (0 as control group, 10, 25 or 50 mg/kg as treatment groups). Thymol was administered every other day 30 min before the subconvulsive PTZ treatment (40 mg/kg, i.p.) for a period of 29 days.

Induction of kindled seizures and behavioral assay

After each PTZ injection, rats were monitored in a glass chamber (30×30×20 cm) and seizure intensity was scored for a period of 30 min. as Racine scale with slight modification [11]: 0=no response; l=mouth and facial clonics; 2=nodding or myoclonic body jerks; 3=forelimb clonus; 4=rearing and forelimb clonus; 5=tonic extension of hindlimb, and status epilepticus. Animals that exhibited phase 4 or 5 in response to first or second PTZ were excluded from the study. Full kindled criteria were considered when the rats exhibited stage 4 or 5 seizures for three times.

Biochemical assay

After behavioral assessment, the rat hippocampi were isolated, blotted dry, then weighed, and prepared as a 5% tissue homogenate in ice-cold 0.9% saline solution. The samples were centrifuged (1000g, 4°C, 10 min.) and the supernatants were aliquoted and stored at -80°C. Microplate reader (BioTek, UK) was used for the biochemical and molecular assessments.

Malondialdehyde assay

The serum concentration of malondialdehyde (MDA), induced by PTZ injection, was evaluated by the thiobarbituric acid reacting substance (TBARS) method. Briefly, MDA reacted with TBARS at a high temperature and produced a red complex TBARS in the supernatant. The absorbance of TBARS was read at 532 nm.

Superoxide dismutase activity assay

Method of Roghani was applied in order to measure superoxide dismutase (SOD) activity [12]. Briefly, the plasma supernatants were incubated by xanthine and xanthine oxidase in potassium phosphate buffer (PH=7.8, 37°C) for 40 min., and then nitroblue tetrazolium (NBT) was added. The resultant dye, blue furmazan, was monitored spectrophotometrically

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at 540 nm. The amount of protein that inhibited NBT reduction to 50% of the maximum was defined as 1 nitrite unit of SOD activity.

TNF-α and IL-1β assay

IL-1β and TNF-α were measured using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems, USA) [13]. The capture antibody of IL-1β was seeded to each well of a 96-well plate for 24 h. Next day, a second set of detective antibody was incubated with the sample tissues or standard antigens in the plate. Thereafter, streptavidin was added. The reaction color converted from purple to yellow, which was recorded at 450 nm.

Statistical analysis

Scores of seizure stages were compared using Kruskal-Wallis (one-way ANOVA on ranks) followed by multiple comparison tests. The level of oxidant indices and inflammatory mediators were compared using fisher ANOVA and tukey's tests. A P value of less than 0.05 was considered to be statistically significant.

Results

According to Racine's standard classification, control rats reached the fully kindled state at 11th PTZ injection. Antiepileptic effect of thymol was stated as the leading factor to retard kindling in the treated rats. For receiving equal number of PTZ injections in the control and treated groups, the treatment with thymol was terminated at 11th PTZ injection. All the kindled rats were introduced to downstream assessments. Repeated injection of PTZ elicited a considerable increase in the levels of MDA ($p<0.01$) and a significant reduction in SOD activity compared to the sham-operated group (Table 1).

Table 1. Effects of thymol on serum malodialdehide levels and SOD activity in rats

Groups	Sal+Veh	PTZ+Veh	Ptz+th10	Ptz+th25	PTZ+Th50
SOD activity (U/mL)	22±3.8	14±2.7*	20±3.1*a	21±2.6*a	18±4.3
MDA (mmol/mL)	6.4±0.25	10.4±0.42	7.1±0.61	7.1±0.25	6.7±0.05

Values are presented as Mean±SD. Sal+Veh: normal saline+vehicle; PTZ+Veh: Pentylenetetrazole+Vehicle; PTZ+Th: PTZ+Thymol at different doses. SOD: superoxide dismutase. MDA: malondialdehide. *means P<0.05 when compared to Sal+Veh. *a means P<0.05 when compared to PTZ+Veh.

TNF-α and IL-1β levels were significantly raised in the control PTZ treated rats. At doses of 10 and 25 mg/kg significantly, thymol decreased the levels of TNF-α ($p<0.001$) and at doses of 5 mg/kg had no significant effects on the level of IL-1β (Fig. 1) and TNF-α (Fig. 2).

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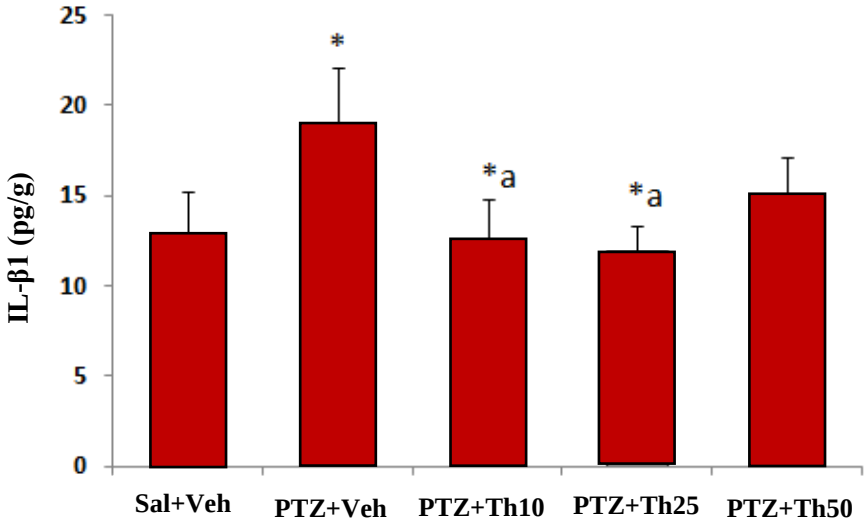


Fig.1. Effect of thymol at different doses on hypocampal level of IL-β1.
* means P<0.05 when compared to Sal+Veh. *a means P<0.05 when compared to PTZ+Veh.

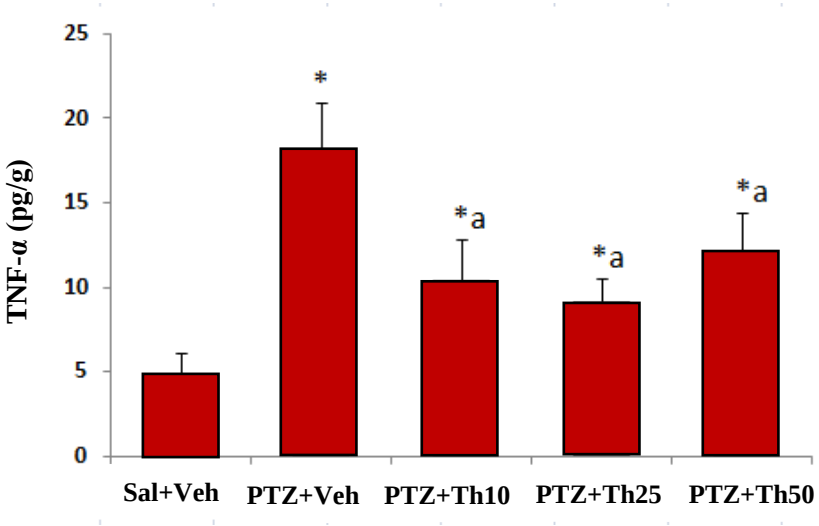


Fig. 2. Effect of thymol at different doses on hypocampal level of TNF-α.
* means P<0.05 when compared to Sal+Veh. *a means P<0.05 when compared to PTZ+Veh.

Discussion

The results of this study reveal that intraperitoneal administration of PTZ can lead to partial and generalized seizures, elevating serum level of MDA and lowering SOD activity. Moreover, repeated administrations of PTZ were able to increase the level of hippocampal proinflammatory cytokines such as TNF-α and IL-1β. Pretreatment of kindled rats with the different doses of thymol retarded the occurrence of advanced epileptic stages, diminished serum oxidative markers including MDA and increased serum SOD activity. Additionally, the hippocampal proinflammatory cytokines such as TNF-α and IL-1β in rats were decreased after pretreatment with thymol. Some studies indicated that PTZ administration caused the level of protein oxidation, lipid peroxidation and inflammatory mediators to increase in the hippocampus and cerebral cortex [10, 14]. In addition, in some regions of

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hippocampus including CA1, CA3 and the dentate hilus KA with activation of ionotropic glutamate receptors could conduct neurons to inadequate O2 utilization, reduced ATP production, inordinate production of ROS, NO, and peroxynitrite with consequent impairment of cell component including lipids, proteins, and DNA. PTZ induction of epilepsy has also been characterized as an inflammatory event [10]. IL-1β and TNF-α are involved in neutrophil migration and inflammatory responses in PTZ-induced inflammation. These mediators are able to recruit leukocytes, such as neutrophils in some inflamed tissues [15, 16]. In this study, thymol showed a significant inhibitory effect on PTZ-induced hippocampal inflammatory response in the epileptic rats. Moreover, the levels of TNF-α, IL-1β were also decreased in thymol treated rats. As a result, a putative anti-inflammatory and in turn anti-epileptic mechanism of thymol could be associated with the degree of inhibition on generation of inflammatory mediators, such as TNF-α, IL-1β. Inflammatory cytokines, including interleukin-1β and TNF-α are released from astrocytes and microglia during and after the seizure induction [17]. IL-1β, in turn, activates IL-1R type I [18]. IL-1R and thus, Toll like receptor signaling change neuronal excitability so that inhibiting excitatory neurotransmission and activating inhibitory neurotrnsmission [19, 20]. TNF-α can stimulate astrocytes to release glutamate [21]. An extracellular increase in glutamate concentration may stimulate glutamatergic neurons, thereby depolarizing their membrane potential. Overall, seizure induced production of inflammatory mediators can be a crucial leading factor in neuronal hyperexcitability through modulations of ion channels and glutamate.

Conflict of Interest

The authors declare that they have no conflict of interests.

Acknowledgments

This research project was supported by deputy of research, Shahid sadoughi university of Medical Sciences, Yazd, Iran. We thanks to the manager and experts of laboratorial center in the Yazd school of medicine for technological assistants.

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