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Acute and subacute effects of thymoquinone on acute methanol intoxication: An assessment based on serum TBARS and BDNF levels in rat model

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ABSTRACT

Objectives: Previous studies have shown the role of oxidative stress in methanol (MeOH) neurotoxicity. In clinical practice ethanol (EtOH) is used for the treatment of MeOH intoxication. Treatment with EtOH results in depression of the central nervous system, which may occur even at therapeutic doses. It also induces oxidative stress. Antioxidant and neuroprotective effects of thymoquinone (TQ) are known in different models of neurotoxicity. There are no studies investigating the protective effect of TQ against acute MeOH intoxication. We aimed to evaluate the effect of TQ administration on serum thiobarbituric acid reactive substances (TBARS) and Brain-Derived Neurotrophic Factor (BDNF) levels in rats with experimentally-induced MeOH intoxication.

Materials and Methods: Six groups were constituted. Methotrexate (Mtx) treatment (0.3 mg/kg/day) intraperitoneally (i.p.) was given for 7 days to slow down the formate metabolism of all rats except controls in order to create a MeOH intoxication similar to that in humans. On the 8th day of the experiment, 3 g/kg MeOH was injected i.p. in MeOH, EtOH and TQ groups. Four hours after MeOH administration, 0.5 g/kg EtOH was injected i.p. in EtOH group and 30 mg/kg TQ was administered i.p. in TQ1 and TQ2 groups. In addition, a total of 5 doses of 30 mg/kg TQ was injected i.p. 24, 48, 72 and 96 hours after the first dose in TQ2 group. Saline solution was given i.p. in the other groups. Blood samples were obtained for evaluating serum TBARS and BDNF levels.

Results: The highest TBARS level was found in MeOH+MTx group and this increase was statistically significant as compared to control and Mtx groups ($p<0.001$). A statistically significant reduction was detected in serum TBARS levels in MeOH+Mtx+EtOH, MeOH+Mtx+TQ1 and MeOH+Mtx+TQ2 groups ($p<0.001$). Maximum serum BDNF level elevation was found in MeOH+Mtx group and this increase was statistically significant as compared to control and Mtx groups ($p<0.001$). Serum BDNF levels were higher in MeOH+Mtx+EtOH, MeOH+Mtx+TQ1 and MeOH+Mtx+TQ2 groups and the difference was statistically significant ($p<0.001$).

Conclusions: Thymoquinone could suppress proinflammation and lipid peroxidation in MeOH intoxication, lead to rapid toxicity adaptation, and play the role of neuroprotection more effectively than EtOH. These results may suggest that TQ could be used as an alternative treatment option in MeOH intoxication.

Keywords: Methanol, Ethanol, Thymoquinone, TBARS, BDNF

1. INTRODUCTION

Methanol (MeOH) is an alcohol that is used as a component of some industrial products such as, perfume, cologne and antifreeze. MeOH intoxication, which may be accidental or intentional as a suicide attempt, can cause severe visual impairment, cerebral infarction and death by the development of formic academia and uncompensated metabolic acidosis [1]. Although, MeOH is not very toxic, it is metabolized into quite toxic substances including formaldehyde and formic acid by alcohol dehydrogenase. Formic acid leads to a reduction in adenosine triphosphate (ATP) synthesis, an elevation in reactive oxygen species (ROS) and cell death directly or through inhibiting cytochrome oxidase in mitochondrial respiratory chain [2]. Treatment is based on the inhibition of alcohol dehydrogenase enzyme that is the first step of conversion to formic acid that is responsible for MeOH intoxication. For this purpose, ethanol (EtOH) and fomepizol are used today of which affinity to alcohol dehydrogenase enzyme is higher than that of MeOH [3].

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Optic nerve damage and basal ganglion necrosis are the well-known side effects of MeOH intoxication. Visual impairment associated with MeOH toxicity begins within 12–48 h due to relatively severe, painless, bilateral optic nerve damage, which may be transient or progressive. Besides, degeneration in cerebellar granular cell layer, an elevation in ROS, a decrease in anti-oxidant capacity, toxic effect of MeOH in rat brain and the reduction in anti-oxidant capacity were shown in autopsy examinations of the cases of MeOH intoxication [4-6].

Thymoquinone (TQ; 2-isopropyl-5 – methyl-1, 4-benzoquinone) is the prime component of the essential oil obtained from Nigella sativa seeds. TQ is a potent antioxidant molecule used as a ROS scavenger for different models of oxidative stress. Also, previous studies have shown anti-inflammatory, immunomodulatory, and neuroprotective effects of TQ in different neurodegeneration and neurotoxicity models. It is thought that TQ's recovery effect on the neural tissue occurs via promotion of neurogenesis and nerve-regeneration, in addition to prevention of neuronal degeneration due to its antioxidant activities [7-10].

Thiobarbituric acid reactive substances (TBARS) are a direct index of cell lipid peroxidation. Brain-Derived Neurotrophic Factor (BDNF) is the most widely distributed neurotrophin in the central nervous system and performs many biological functions such as neural survival, differentiation and plasticity [11, 12]. Neurotrophins, antioxidant enzymes and oxidative markers have complex and reciprocal interactions. Therefore, utilization of antioxidant and antiinflamatory defense strategies may counterbalance oxidative damage and thereby ameliorate neurotrophic imbalance and signaling to protect neuronal cell. A growing number of experimental evidence have emerged, which support the concept that TQ with that strong antioxidant and anti-inflamatory activity ameliorates apoptosis, neurotrophic factors and oxidative stress and thereby may prevent damage to the neuronal tissue [11, 12].

Compared to humans, liver folate content of rats is higher and their folate metabolism is faster. Therefore, it is difficult to detect formic acid accumulation and metabolic acidosis. Experimental studies have shown that Methotrexate (Mtx) reduces folate content in rats. Therefore, Mtx is used to slow down the formate metabolism of rats in order to create a MeOH intoxication similar to that in humans.

Current research revealed that acute MeOH intoxication leads to effects very rapidly and in a very short duration. All this information suggests that TQ will provide good results for the treatment of acute MeOH intoxication. There are no studies investigating the protective effect of TQ against acute MeOH intoxication in the literature. To the best of our knowledge, this is the first study in literature investigating acute and subacute effects of TQ and EtOH treatments following acute MeOH intoxication on neurotrophic factor and oxidative stress levels through serum TBARS and BDNF levels.

2. MATERIALS and METHODS

This study was approved by the Necmettin Erbakan University KONUDAM Experimental Medicine Application and Research Center (Approval number: 68).

Animals

A total of 52 albino Wistar Albino male rats weighing 280-320 were used in the experiments. Animals were kept and fed at normal room temperature (22°C) prior to the experiment.

Chemicals

Methotrexate (Mtx, Koçak Farma, Istanbul, Turkey) was diluted in saline. MeOH, EtOH and TQ were purchased from Sigma Chemical Co (St. Louis, MO, USA). MeOH and EtOH were diluted in saline, and administered as a 20% w/v solution. TQ was dissolved in EtOH with further dilution in 0.9 % saline.

Experimental groups

This study included six groups, each containing nine rats except control group. The control group had seven rats. The groups were control, Methotrexate (Mtx), Mtx+MeOH, Mtx+MeOH+EtOH, Mtx+MeOH+TQ1, Mtx+MeOH+TQ2. The number of animals in the groups was determined by considering the necessity of using the minimum number of animals necessary for statistical significance.

Experimental procedure

Liver folate content is higher and folate metabolism is faster in rats as compared to humans. It is therefore difficult to develop formic acid accumulation and metabolic acidosis. Mtx was shown to reduce folate content in rats in experimental studies [5]. Therefore, all rats except controls were administered Mtx (0.3 mg/kg/day) intraperitoneally (i.p.) for 7 days for developing MeOH intoxication in rats similar to humans and for slowing formate metabolism. On the 8th day of the experiment, i.p. injection of MeOH (3 g/kg) was administered in MeOH, EtOH, TQ groups. Four hours after MeOH treatment, 0.5 g/kg EtOH was injected i.p. in EtOH group; 30 mg/kg TQ i.p. in TQ1 and TQ2 groups. In addition, a total of 5 doses of 30 mg/kg TQ was injected i.p. at 24, 48, 72 and 96 hours after the first dose in TQ2 group. Saline solution was given i.p. in the other groups. Rats were sacrificed 8 hours after the administrations with 50 mg/kg ketamin HCl anesthesia. Blood samples were obtained from the animals in order to determine serum TBARS and BDNF levels.

Biochemical analysis

Venous blood samples were collected by centrifugation at 4 ^{0}C and 1000 g for 10 minutes to separate serum. Serum samples were stored at – 80 ^{0}C until the start of the experiment. Serum TBARS (Oxford Biomedical Research, Kansas City, Missouri, USA) levels were measured using Enzyme-Linked Immuno Sorbent Assay (ELISA). The results were expressed in nmol/mL. Serum BDNF (Boster Biological Technology, CA,USA) levels were measured and the results were expressed in pg/mL.

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Statistical Analysis

The Kolmogorov-Smirnov test was used for the parametric distribution of numerical parameters. All data are expressed as mean ± standard error of the mean (x ± SEM). Biochemical results were analyzed using SPSS version 20 software (SPSS, Chicago, IL, USA). Difference of variances between the groups was analyzed by ANOVA followed by post-hoc Tukey test. Pearson's correlation test was used for correlation of numerical values. Statistical significance was declared at p<0.05.

3. RESULTS

At the end of the study, all rats were evaluated without any failure. Serum TBARS and BDNF levels of the groups are presented in Table I.

Table I. *Comparison of serum BDNF and TBARS levels*

Groups		BDNF (pg/mL)	TBARS (nmol/mL)
I-Control		86.82±4.53	2.55±0.17
II-Mtx		183.23±16.64	3.54±0.22
III-MeOH+Mtx		229.56±15.20	6.08±0.67
IV-MeOH+Mtx+EtOH		150.64±12.24	5.41±0.59
V-MeOH+Mtx+TQ1		129.52±10.32	3.06±0.25
VI-MeOH+Mtx+TQ2		106.09±11.02	2.30±0.22
P values			
I-II		<0.001	<0.001
I-III		<0.001	<0.001
I-IV		<0.001	<0.001
I-V		<0.001	<0.001
I-VI		<0.001	<0.001
II-III		<0.001	<0.001
III-IV		<0.001	0.040
III-V		<0.001	<0.001
III-VI		<0.001	<0.001
IV-V		0,001	<0.001
IV-VI		<0.001	<0.001
V-VI		<0.001	<0.001

The highest TBARS level was detected in MeOH+Mtx group and this elevation was statistically significant as compared to control and Mtx groups (p<0.001). A reduction was detected in TBARS levels in treatment groups (MeOH+Mtx+EtOH, MeOH+Mtx+TQ1, MeOH+Mtx+TQ2) as compared to intoxication group (MeOH+Mtx) and the difference was statistically significant (p:0.040, p<0.001 and p<0.001, respectively). TBARS levels in groups MeOH+Mtx+TQ1 and especially MeOH+Mtx+TQ2 were found to be similar with those in control group. The reduction in TBARS levels in MeOH+Mtx+TQ2 group as compared to MeOH+Mtx+TQ1 group was statistically significant (p<0.001).

Maximum serum BDNF level elevation was found in MeOH+Mtx group and this increase was statistically significant as compared to control and Mtx groups (p<0.001). Serum BDNF levels were statistically significantly higher in MeOH+Mtx+EtOH, MeOH+Mtx+TQ1 and MeOH+Mtx+TQ2 groups as compared to control (p<0.001). A decrease was detected in serum BDNF levels in treatment groups (MeOH+Mtx+EtOH, MeOH+Mtx+TQ1, MeOH+Mtx+TQ2) as compared to intoxication group (MeOH+Mtx) and the difference was statistically significant (p<0.001). Despite the statistically significant elevation in serum BDNF levels in MeOH+Mtx+TQ1 and particularly in MeOH+Mtx+TQ2 groups (p<0.001), especially in the MeOH+MTX+TQ2 group, the serum BDNF level was closest to the control group.

Pearson's correlation analysis revealed a strong positive correlation between TBARS and BDNF (r:0.773, p:<0.001;Figure 1).

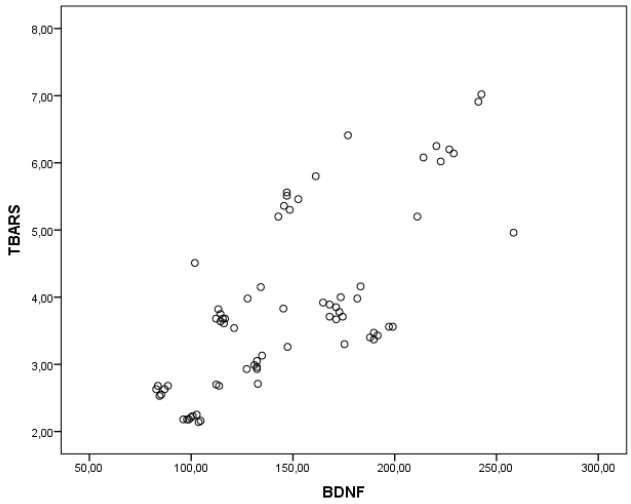

Figure 1. *Pearson's correlation coefficient (r) and p-value (p) between TBARS and BDNF (r:0.773 p:<0.001)*

4. DISCUSSION

In this experimental study, we have investigated the role of acute and subacute effects of TQ on serum TBARS and BDNF levels in rat model with acute MeOH intoxication. It has been concluded that TQ administration could significantly suppress proinflammation and lipid peroxidation that occur during MeOH intoxication, and may also lead to rapid toxicity adaptation.

Although, MeOH is not very toxic, it is metabolized into quite toxic substances including formaldehyde and formic acid by alcohol dehydrogenase. Formic acid leads to a reduction in ATP synthesis, an elevation in ROS and cell death directly or through inhibiting cytochrome oxidase in mitochondrial

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respiratory chain [2]. ROS are continuously produced during normal physiologic events and removed by antioxidant defense mechanism. The imbalance between ROS and antioxidant defense mechanisms leads to lipid peroxidation and oxidative damage in the lipid bilayers surrounding both the cell itself and membrane-bound organelles. Rajamani et al., suggested that MeOH exposure results in increased free radical generation and significant protein oxidative damage in the retina and optic nerves of the rats [5].

Thiobarbituric acid reactive substances are a direct index of cell lipid peroxidation [11]. In our study, we have detected a significant elevation in serum TBARS level that is a standard marker for lipid peroxidation, particularly in intoxication group (MeOH+Mtx). A reduction was detected in TBARS levels in treatment groups (MeOH+Mtx+EtOH, MeOH+Mtx+TQ1, MeOH+Mtx+TQ2). In the MeOH+MTX+TQ1 and especially in the MeOH+MTX+TQ2 group, TBARS levels were almost similar to the control group. In the study plan, single dose of TQ was given to MeOH+Mtx+TQ1 group and acute effects were tried to be measured. On the other hand, a total of 5 doses of TQ were given to MeOH+Mtx+TQ2 group with 24 hours of intervals and subacute effects were tried to be measured. Results of the study reveal that TQ treatment is effective in both acute and even more evident in subacute period through effectively reducing TBARS levels. The present study also reveals that TQ treatment is quite effective for preventing lipid peroxidation and this effect is more evident in MeOH+Mtx+EtOH group. Given that this effect continues even at the end of 96th hour that may be defined as subacute period, it may be suggested that TQ treatment could be a strong lipid peroxidation preventer in MeOH intoxication that may continue its toxic effect between 12 and 48 hours. Administration of TQ in subacute period has not been encountered in literature. We would like to state that the result of the study is an important stage for treatment stages of MeOH intoxication that lead to severe and irreversible damage particularly in neuronal tissue [1,5,13].

Brain-derived neurotrophic factor is the most ubiquitous and intensively studied member of the family of neurotrophins in the central nervous system (CNS). In our study, maximum serum BDNF elevation was detected in MeOH+Mtx group. Serum BDNF levels decreased in treatment groups (MeOH+Mtx+EtOH, MeOH+Mtx+TQ1, MeOH+Mtx+TQ2) and BDNF levels in MeOH+Mtx+TQ2 group were similar to those in control group. In a study, evidence was reported that the elderly women with acute low back pain exhibited higher plasma BDNF levels as compared to the subjects without pain and the researchers stated that pro-inflammation in the low back pain group could increase BDNF protein expression [14]. In another study, plasma BDNF levels of metamphetamine users were found to be significantly higher than controls and the researchers stated that this result provided evidence that BDNF played an important role for neuro-adaptation against metamphetamine neuro-toxicity [15]. In our study, the significant increase in BDNF levels in MeOH+Mtx group may suggest toxicity adaptation against acute MeOH toxicity and that acute-toxicity-induced pro-inflammation increased BDNF protein expression.

The significant decrease in BDNF levels in treatment groups (MeOH+Mtx+EtOH, MeOH+Mtx+TQ1, MeOH+Mtx+TQ2) as compared to MeOH+Mtx group may indicate that toxicity adaptation rapidly develops through EtOH and TQ treatments and also pro-inflammation is suppressed. Given the subacute course of the treatment particularly in MeOH+Mtx+TQ2 group and the similar BDNF values with control group, it may be considered that aforementioned potential mechanisms work more effectively.

Brain-derived neurotrophic factor is associated with several processes that are essential for the optimal functioning of especially neural tissues. Several studies reported altered brain and plasma BDNF levels in patients with various brain pathologies [16]. However, the pathophysiological mechanisms that underlie these changes are not yet fully understood. There is also no conclusive evidence that can discriminate whether changes in BDNF levels are a causative or the consequence of the disease onset. Neuro-inflammation is regulated by factors that are also involved in modulation of BDNF expression. Both neuro-inflammation and altered BDNF expression are common phenomena in many disorders especially neural disorders. The optic nerve, retina, and basal ganglia are the main tissues that are at risk from MeOH intoxication which may be associated with the high mitochondrial energy requirement in these tissues. Remarkably, there are only few studies that have investigated the link between BDNF and neuro-inflammation. Better understanding of the interaction between BDNF and neuro-inflammation could open new ways for therapy management and could facilitate the development of new therapeutic strategies for neurological diseases [16].

Pearson's correlation analysis revealed a strong positive correlation between TBARS and BDNF in the present study (r:0.773 p:<0.001). A positive correlation was detected between TBARS and BDNF in the previous *in vivo* and *in vitro* studies. In an *in vivo* study, elevated BDNF levels were explained with compensation mechanism of the metabolic stress and related oxidative damage [17-19]. The positive correlation between TBARS and BDNF suggest that further studies are required about the intra-cellular interactions of neurotrophins, anti-oxidant enzymes and oxidative markers.

No studies investigating the protective effect of TQ against acute MeOH intoxication were found in the literature. Studies have shown anti-inflammatory, immunomodulatory, and especially neuroprotective effects of TQ in different models of neurodegeneration and neurotoxicity. It is thought that TQ's recovery effect on the neural tissue occurs via promotion of neurogenesis and nerve-regeneration, in addition to prevention of neuronal degeneration due to its antioxidant and antinflammatory activities. In the present study, it was aimed to reveal that TQ treatment could be effective both in acute and subacute processes of MeOH intoxication through serum TBARS and BDNF levels. As a result, it was concluded that TQ administration could suppress proinflammation and lipid peroxidation occurring in acute and subacute periods of MeOH

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intoxication, lead to rapid toxicity adaptation and perform it more effectively than EtOH treatment. These results may show that TQ could be used as an alternative treatment in MeOH intoxication. Also, further studies that examine serum, tissue and histopathological data together are required in order to clearly reveal the effects of TQ treatments on MeOH metabolism.

Compliance with Ethical Standards

Ethical Approval: This study was approved by Necmettin Erbakan University KONUDAM Experimental Medicine Application and Research Center (Protocol number: 68). All methods were performed in accordance with the relevant guideline regulations.

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Conflict of interest: The authors have no potential conflicts to declare.

Author Contributions: HHK: Design, HHK and IK: Supervision, HHK, IK and AO: Resources, materials and collection and/or processing, HHK and IK: Analysis and/ or interpretation, HHK and IK: Writing the article, HHK, IK, and AO: Critical review.

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