Intraperitoneal injection of buprenorphine on anxiety-like behavior and alteration in expression of *Gfap* and *Nrf2* in methamphetamine treated rats

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Article Info	Abstract
	The effects of buprenorphine (BUP) on anxiety-like behavior and the expression of the glial fibrillary acidic protein (*Gfap*) and nuclear factor erythroid 2–related factor 2 (*Nrf2*) in methamphetamine (METH)-treated rats were investigated in this study. Twenty-eight male Wistar rats were randomly divided into four groups including control (saline), METH (10.00 mg kg^{-1}), BUP (10.00 mg kg^{-1}), and BUP+METH groups and treated for five days. On the final day of treatment, gene expression levels and anxiety were evaluated using elevated plus-maze (EPM). According to the results, five days of METH injection reduced open arm exploration in the EPM. In contrast, the open arm entries and the time spent in the open arms were increased in the BUP+METH group compared to the METH group. The expression levels of *Gfap* and *Nrf2* were lower in METH-treated rats compared to controls, whereas *Gfap* and *Nrf2* expression levels were higher in the METH+BUP-treated rats compared to the METH-treated rats, however, it was similar to the controls. These findings suggested that co-administration of BUP+METH could decrease anxiety-like behavior through increasing the activity of the antioxidant protection system and might have therapeutic potential for preventing anxiety in METH users. © 2022 Urmia University. All rights reserved.

Introduction

According to the latest data, about 40.00% of meth-amphetamine (METH) users suffer from anxiety disorders.[1,2] The therapeutic approaches aiming at preventing anxiety following METH dependence have focused on the neurobiological mechanisms of this disorder using combination of behavioral, electrophysiological and molecular techniques.[3,4]

Several studies indicated the effectiveness of buprenorphine (BUP) in the treatment of anxiety, other behavioral disorders, opioid abuse and even in anxiety-refractory cases.[5] Several studies examined the associations between psychostimulants and opioid receptor agonists.[6] Treatment with BUP reduced psychostimulant self-administration-induced in rhesus monkeys[7] by modulating dopaminergic neurotransmission.[8] On the contrary, it was stated that treatment with BUP damaged brain tissue and decreased number of neurons.[9]

The METH exposure resulted in mitochondrial oxidative damage.[10] Nuclear factor erythroid 2-related factor 2 (*Nrf2*) is a crucial regulator of cellular resistance to oxidants.[11] Its protective activities are not only restricted to antioxidative transactivation and it plays a critical role in encountering different physiological and pathological stresses.[12] Furthermore, the latest studies emphasized the association between oxidative stress and anxiety or the possible causal relationship between them.[13,14]

Besides, long-term METH exposure induces eminent activation of glial fibrillary acidic protein (*Gfap*)[15] as an astrogliosis marker in the cortex and hippocampus.[16] Studies have shown that the activated microglia and astrocytes, through the creation of pro-inflammatory cytokines, are associated with METH-induced inflammation and neurodegeneration.[17] It is generally believed that METH induces oxidative stress, which in turn can increase pro-inflammatory cytokines.[10] It was

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reported that chronic inflammation in the central nervous system could regulate anxiety.[18]

The METH-related anxiety is a complex disease which its underlying molecular pathway is poorly understood. No studies have been found to evaluate the effects of METH on anxiety through *Gfap* and *Nrf2* mRNA expression. In addition, there are paradoxical reports on the impact of different doses of METH on the nervous system[3] or additional effects of BUP on anxiety.[19] Therefore, the present research evaluated the effects of BUP on anxiety-like behavior in METH-treated rats based on the results of *Gfap* and *Nrf2* expression.

Materials and Methods

Animals. Twenty-eight adult male Wistar rats (220 ± 30.00 g) were obtained from the Tehran Institute, Iran. The animals were kept in a room with a controlled temperature under a 12:12 hr light/dark cycle (lights on 7:00 a.m. to 7:00 p.m.). The rats were given *ad libitum* access to food and water. They were adapted to the research environment for at least one week prior to the tests. Animal protocols were approved by the Ethics Committee of Shahrekord University of Medical Sciences (IR.SKUMS.REC.1398.194). All procedures for the main-tenance and use of the experimental animals were conducted in accordance with the guide for the care and use of the laboratory animals (NIH Guide for Care and Use of Laboratory Animals, 8th ed., 2011).

Drugs. The METH was purchased from Sigma-Aldrich (St. Louis, USA) and dissolved in normal saline (Shahid Ghazi, Tabriz, Iran). The BUP was prepared from Faran Shimi (Tehran, Iran) and dissolved in normal saline.

Study groups. Twenty-eight rats were randomly assigned to four groups (n = 7) and intraperitoneal injections including the control (normal saline; 1.00 mL kg⁻¹), METH (10.00 mg kg⁻¹),[20] BUP (10.00 mg kg⁻¹),[21] and combination of BUP (10.00 mg kg⁻¹) and METH (10.00 mg kg⁻¹) groups and treated for five days.[12] After treatments, the animals were subjected to the elevated plus-maze (EPM) task for behavioral changes.

Elevated plus-maze (EPM) test. The EPM device was composed of two open and two closed arms (length: 51.00 and width: 10.00 cm) passing in the middle and perpendicular to each other forming a plus shape. The maze was elevated 50.00 cm above the ground. Each rat was put in the center of the maze and allowed to explore all arms freely and the behavior was controlled for 5 min over the maze using a digital camera. The device was cleaned with 10.00% ethanol after each experiment to remove any odors. The time spent in the open arms as well as the number of entries into the open arms was calculated. The total number of entries into the open and close arms represented the distance traveled by animals.[22]

**Real time-polymerase chain reaction (RT-PCR) technique.** The rats were rapidly decapitated 24 h after the behavioral experiments and the cerebral cortex was removed and frozen in liquid nitrogen instantly. Using the YTzol Pure RNA buffer (Yekta Tajhiz, Tehran, Iran), total RNAs were extracted from the cerebral cortex samples. Then, their concentration and purity were detected by the NanoDrop instrument. Finally, using the reverse transcription kit (Bioneer, Daejeon, South Korea), the RNA was exposed to reverse transcription. Triple reactions were used to measure mRNA expression levels of *Gfap* and *Nrf2* in cDNA samples via the gene-specific primers (*Gfap:* forward 5'-GAGCCAAGGAGCCCACCAAAC-3' and reverse 5'-GATTGT CCCTCTCCACCTCCA-3'; *Nrf2:* forward 5'-GTGGCTTACA ACGGACATGGA-3' and reverse 5'-GGAGTTGCTCTTGT CTCTCCT-3'; *Gapdh:* forward 5'- CTCTCTGCTCCTCCCTG TTCT -3' and reverse 5'- CGTCCTTCCCCCCATTCCTAA-3'). The relative expression of the genes was then assessed. In the next step, RT-PCR was done by the SYBR Green PCR Master Mix (Takara Bio USA, Inc., San Jose, USA). Ultimately, quantitative real-time PCR was done using the comparative ΔΔCT Method, and also, an arithmetic formula was employed to measure the relative expression of the target mRNAs in relation to the reference values.[23]

Statistical analysis. Data were presented as mean ± SEM and analyzed by SPSS Software (version 21.0; IBM Corp., Armonk, USA). One-way ANOVA and Tukey's post-hoc test were applied to analyze the behavioral experiment (EPM) results or determine the expression levels of *Gfap* and *Nrf2* for intergroup comparisons. A $p < 0.05$ was considered statistically significant.

Results

Effects of METH and BUP administration on animals in the elevated plus-maze test. Figure 1 shows the effect of treatments on animals in the EPM test. The results of one-way ANOVA showed a significant difference in the number of entries into the open arms between groups [$F_{(3, 24)} = 15.01$, $p < 0.001$]. Also, there was a significant difference in the time spent on the open arms between groups [$F_{(3, 24)} = 14.72$, $p < 0.001$]. The rats in the METH group showed a significant reduction in the number of open arms entries (1.14 ± 0.55; $p < 0.01$) and time spent (21.71 ± 4.96; $p < 0.001$) in the open arms compared to the number of entries (7.28 ± 0.74) and spent time in the open arms (50.85 ± 2.89) in the control group. In the BUP group, the open arm entries (11.71 ± 2.01) and time spent in these arms (55.85 ± 3.05) were not significantly different compared to the control rats, however, they showed a significant increase in the open arm entries compared to the METH group ($p < 0.001$).

Compared to the METH group, the number of entries into the arms and time spent in open arms in the BUP+METH group were changed. BUP+METH co-administration led to a significant increase in the number of entries into the open arms (8.28 ± 0.81) and time spent in these arms (42.28 ± 4.38) compared to the number of entries (1.14 ± 0.55; $p < 0.001$) and time spent in open arms (21.71 ± 4.96; $p < 0.01$) in the METH group (Fig. 1).
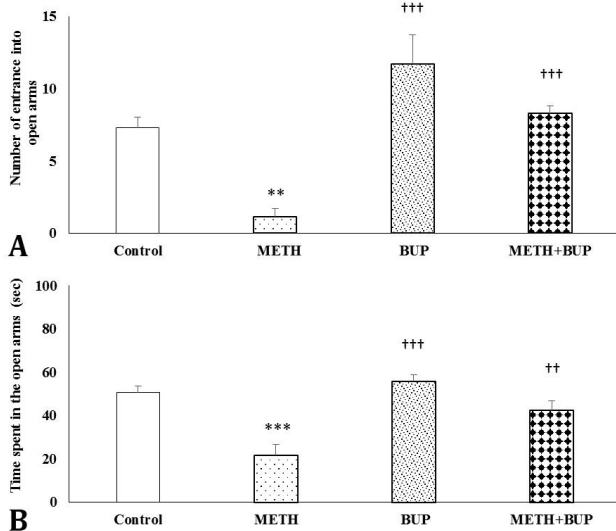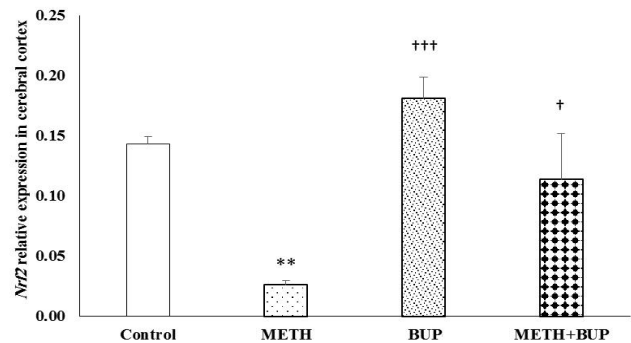

Fig. 1. Effects of intraperitoneal methamphetamine (METH), buprenorphine (BUP) injection, and their co-administration (BUP+METH) on **A)** number of entries into open arms and **B)** time spent in the open arms in the elevated plus-maze (EPM) test. ** $p < 0.01$ and *** $p < 0.001$ compared to the control group and †† $p < 0.01$ and †††$p < 0.001$ compared to METH-treated group. Data are represented as mean ± SEM.

Figure 2 shows the finding of total traveled distance in the EPM. The One-way ANOVA results showed that there was no significant difference in the total number of entries into the open and close arms among the experimental groups [F (3, 24) = 1.83, $p = 0.17$].
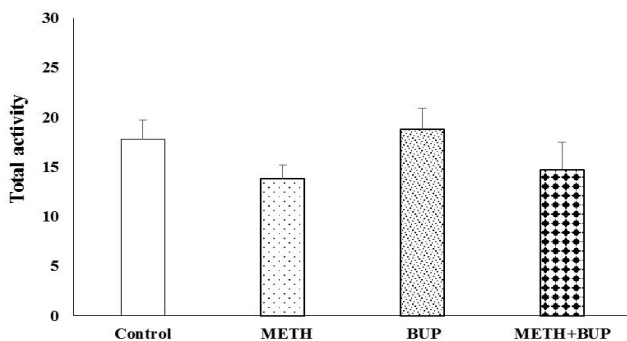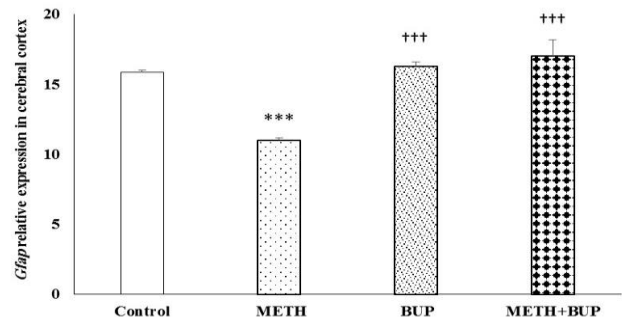

Fig. 2. The effects of intraperitoneal methamphetamine (METH) and buprenorphine injection (BUP) and their co-administration (BUP+METH) on traveled distance in the elevated plus maze (EPM). Total activity, the mean total number of entries into the open and close arms, was measured. The groups did not exhibit any differences ($p > 0.05$). Data are represented as mean ± SEM.

The expression level of *Nrf2* and *Gfap* in response to METH and BUP administration. Effects of BUP on the cerebral cortex *Gfap* and *Nrf2* mRNA levels following the administration of METH and BUP were tested by RT-PCR. According to the results, the *Nrf2* mRNA expression levels was significantly decreased in the METH group compared to control rats ($p < 0.01$), whereas, *Nrf2* gene expression showed a significant increase in the BUP group compared to the METH group ($p < 0.001$). The BUP+METH group showed a significant increase in the expression levels of *Nrf2* than the METH group ($p < 0.05$) [*Nrf2* mRNA levels: METH: 0.03 ± 0.00 BUP: 0.18 ± 0.17; METH + BUP: 0.11 ± 0.38; and control: 0.14 ± 0.00; Fig. 3).

Fig. 3. The effects of intraperitoneal methamphetamine (METH) and buprenorphine (BUP) injection and their co-administration (BUP+METH) on the cerebral cortex *Nrf2* mRNA levels. ** $p < 0.01$ compared to the control group and † $p < 0.05$ and ††† $p < 0.001$ compared to the METH-treated group. Data are represented as mean ± SEM.

Also, the METH group exhibited a significant decrease in *Gfap* mRNA levels than controls ($p < 0.001$). The BUP+METH ($p < 0.001$) and BUP ($p < 0.001$) groups showed a significant increase in the *Gfap* mRNA levels compared to METH rats. The *Gfap* mRNA levels were as follow. METH: 10.99 ± 0.18; BUP: 16.28 ± 0.29; METH + BUP: 17.03 ± 1.13; and Control: 15.85 ± 0.14, (Fig. 4).

Fig. 4. The effects of intraperitoneal methamphetamine (METH) and buprenorphine (BUP) injection, and their co-administration (BUP+METH) on the cerebral cortex *Gfap* mRNA levels. *** $p < 0.001$ compared to the control group and ††† $p < 0.001$ compared to the METH-treated group. Data are represented as mean ± SEM.

Discussion

This study evaluated the effect of a 5-day co-administration of BUP plus METH on anxiety behavior and alteration in the expression levels of *Nrf2* and *Gfap* in the METH-treated rats. The main findings were as follow: 1) Treatment of rats with METH considerably reduced the number of entries into the arms as well as the time spent in open arms compared to the control group in EPM, 2) These parameters significantly were increased after the administration of METH + BUP (10.00 mg kg⁻¹) in comparison with the METH group, and 3) There was a correlation between anxiety and alterations in the expression levels of *Nrf2* and *Gfap*.

Previous studies showed that different doses of psycho-stimulants had diverse effects on the responses to behavioral parameters[24] and the levels of brain neuro-trophic factors which were associated with the applied concentration, injection type and duration of treatment.[3,25] The present research, for the first time, evaluated the effect of high-dose METH using EPM in which a 5-day injection of METH decreased the number of entries into the open arms and the time spent in the open arms in the studied subjects. Some studies have shown that treatment with METH-induced severe self-injurious behavior with-out affecting locomotor activity.[26] It has also been revealed that administration of low doses of METH to the METH-sensitized rats caused a reduction or an increase in the time spent in open arms and open arm entries at 30 or 120 min after injection compared to the control group, respectively.[27,28] Also, the significant reduction in the level of norepinephrine metabolite was associated with anxiety-related behavior observed in the EPM.[29]

Our findings demonstrated that the administration of BUP increased the number of open arm entries and the time spent compared to MTEH rats. Therefore, BUP at 10.00 mg kg⁻¹ caused anxiolytic effects in rats. The BUP is used to treat anxiety-related behaviors.[30,31] The findings of another study demonstrated that the anxiety-like behaviors caused by BUP were due to its antagonist properties that interfered with the δ opioid receptor and due to lack of agonist activity on kappa (κ)-opioid receptor.[31] On the other hand, U-50 488H, a κ-opioid-receptor agonist had anxiolytic-like effects.[32] Accordingly, BUP has strong and permanent antidepressants effects and also affects anxiety, as well.

Administration of BUP to METH-treated rats resulted in anxiolytic effects because the rats showed a significant increase in open arm exploration compared to the METH group in the EPM. This finding was not in agreement with that reported by Etaee *et al.*,[33] who suggested co-administration of METH and BUB increased anxiety.[30] These conflicting results are likely due to differences between the protocols of the administration as well as and the doses of METH and BUP used in the experiments.

Our result showed that exposure to METH significantly decreased *Nrf2* expression compared to the other groups. Injection of METH was found to cause oxidative stress which was correlated with changes in the levels of *Nrf2* as a main regulator of redox homeostasis in anti-oxidative responses.[11] In work of others, administration of low doses of METH increased *Nrf2* gene expression in astrocytes, whereas, it did not affect the number of neurons, which was not consistent with our results.[34] Another report showed that the role of *Nrf2* gene deletion in METH-induced oxidative stress could be partially compensated by other mechanisms in which *Nrf2* was not involved.[35]

Previous animal studies showed a robust activation of both astrocytes and microglia after exposure to a neuro-toxic regimen of METH [15,36,37] and another investigation demonstrated reactive microglia and increased density of *Gfap*-positive astrocytes in the brains of human METH abusers.[15,38] Inconsistently, animal studies revealed that a single dose METH administration at 10.00 or 20.00 mg kg⁻¹ did not significantly increase *Gfap*, while, METH administration at 30.00 and 40.00 mg kg⁻¹ increased *Gfap* dose-dependently.[39] The reason for this difference could be due to the protocols of the administration, duration of addiction and METH dosage in the experiments.

Consistent with the mentioned mechanisms, glial activation potentiated by *Nrf2* deficiency has been reported one day following METH administration by enhanced striatal expression of astroglia markers (*Gfap*).[40] To the best of our knowledge, there are no studies on the effect of BUP on the alterations in the expression of the *Nrf2* gene and *Gfap* which indicates the novelty of our research. Our findings exhibited that co-administration of BUB plus METH significantly increased the *Gfap* gene expression in comparison with the METH treatment. Several studies have reported a strong activation of astrocytes[36] and microglia[37] following exposure of animals to a single injection of METH. However, other findings showed that animals resistant to neurotoxicity caused by the acute injection of METH showed no significant microglial activation after the re-exposure which was similar to the results where the animals were exposed to the acute toxicity.[15,37]

In summary, the present study showed that co-administration of BUP and METH could decrease anxiety behavior through the increased activity of the antioxidant system and it might have therapeutic potential for preventing anxiety in METH users. Additional histological experiments are needed in the future to elucidate the involved mechanisms.

Acknowledgments

Conflict of interest

The authors declare that they have no conflict of interest.

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