



Anti-nociceptive and antioxidant activity of betaine on formalin- and writhing tests induced pain in mice

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ABSTRACT

Pain is a physiological response which is mediated via the central and peripheral nervous system. Betaine, is a methyl glycine derivative and a commonly used nutrient supplement. The main purpose of the current paper is to determine the possible anti-nociceptive and antioxidant activity and sedative effect of betaine in mice. Adult male albino mice were divided into two categories, formalin and writhing tests. In the formalin test, mice were injected with betaine (10, 20 and 30 mg/kg) or morphine (5 mg/kg). For co-injections mice received betaine (30 mg/kg) + naloxone (2 mg/kg) or atropine (1 mg/kg), chlorpheniramine (20 mg/kg), flumazenil (5 mg/kg), cimetidine (12.5 mg/kg) and cyproheptadine (4 mg/kg). Then the formalin test was done and paw licking time was determined. In the writhing test, injections were the same but the animals were injected with acetic acid (0.6 %) and the percentage of writhing inhibition was recorded. At the end of the study, blood antioxidant levels were determined. According to the results, betaine reduced the pain response in a dose-dependent manner. Co-administration of the naloxone + betaine or flumazenil + betaine significantly decreased the anti-nociceptive effect of betaine on the licking and biting time of the injected paw and inhibited the number of writhing movements. Betaine decreased malondialdehyde (MDA) and improved superoxide dismutase (SOD) and glutathione peroxidase (GPx) levels in formalin receiving mice. No adverse locomotion and sedation effect were observed in betaine-treated mice. These findings suggest that betaine has anti-nociceptive and antioxidant activity in mice, and its anti-nociceptive role interacts with opioidergic and GABA receptors.

1. Introduction

Pain is a sensorial modality and is primarily protective in physiological systems, but often leads to discomfort [1]. Visceral pain such as angina, colic, dyspepsia, pancreatitis, appendicitis and dysmenorrhea is mediated via visceral nociceptors [2]. Approximately 4–40% of adults around the world suffer from chronic pain resulting from disease and medical conditions [3]. Inflammatory pain is a kind of chronic pain which is accompanied by release of inflammatory mediators from damaged tissues [4]. Interaction of inflammatory mediators with nociceptors increases pain transmission and readies the nervous system for nociception [5].

Several neurotransmitters such as opioids, γ -aminobutyric acid (GABA), noradrenaline and histamine have a mediatory role on nociception. In the central nervous system (CNS) and peripheral neurons system (PNS), opioid receptors are responsible for opioid-induced analgesia during painful stimuli and inflammatory and neuropathic pain [6]. Management of chronic pain, is more challenging and less effective compared to the acute pain, due to physio-psychological complications

[7]. Due to the side effects of analgesics agents such as opioid analgesics and nonsteroidal anti-inflammatory drugs, there is a growing interest for novel natural analgesic agents with fewer side effects [2].

Betaine was first discovered from the *Beta vulgaris* plant and has been isolated from numerous microorganisms, plants and animals [8]. Betaine is synthesized in choline-mediated one-carbon metabolism and is known as a key molecule in the methionine/homocysteine cycle [9], cell signaling and neurotransmitter synthesis [10]. In vertebrates, betaine is ingested from the diet as well as endogenously synthesized in mitochondria from its precursor choline using choline dehydrogenase [12]. Daily intake of 9–15 g betaine is safe, and it is distributed in the human liver, kidneys and brain [11]. Several biological functions have been proposed for betaine, such as osmotic regulator; antioxidative/anti-inflammatory activity; supplier of methyl donor S-adenosylmethionine; and mitigator of noxious elevated homocysteine [9]. The latter two are endowed through acceleration of the turnover of the methionine–homocysteine cycle (constituting one-carbon metabolism together with the folate cycle), where betaine serves as a substrate in the betaine–homocysteine S-methyltransferase (BHMT) reaction,

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converting homocysteine to methionine [10]. Betaine is known to act against various biological stresses and its levels were reported to be decreased in schizophrenia patients [12,13]. Betaine gamma amino butyric acid transporter (BGT-1) and amino acid transport system A are the main transport systems of betaine in most tissues [14]. Betaine was shown to play a major role in improvement of individuals suffering from mild-to-moderate depression [9] as injection of 30 and 100 mg/kg betaine in rats decreased the immobility time in forced swimming tests [14]. Betaine-induced alterations in serotonin levels during forced swimming tests were observed in the hippocampus and hypothalamus [15]. It has been reported that injection of betaine (0.163 mmol/kg, s.c.) also prevented memory impairment [14].

It has been reported that dietary choline and betaine intake has an anti-inflammatory effect [16,17]. Taking dietary betaine (1, 5 and 10 mg/kg) for 16 weeks effectively suppressed the expression of TNF- α , IL-6, COX-2 and iNOS in mouse colon tumors [18]. Recently, it was reported that betaine has antioxidant activity which decreases MDA levels while increasing SOD and GPx levels in generation of reactive oxygen species (ROS) [11]. According to the literature, there is scarce information about the bioactivity of betaine. Therefore, the primary purpose of the current paper was to determine the anti-nociceptive effects of betaine by formalin and writhing tests [18]. Also, the secondary outcome was to determine the antioxidant activity of betaine as well as its effect on locomotion. The third purpose of the study was to determine the possible interaction of the anti-nociceptive activity of betaine with opioidergic, histaminergic, muscarinergic, GABAergic and serotonergic systems.

2. Material and methods

2.1. Animals

In this study 384 adult male albino Naval Medical Research Institute (NMRI) mice (25–30 g) were prepared (Pasteur Institute, Tehran, Iran) and kept in groups of 8–10 per cage (45 cm \times 30 cm \times 15 cm) at (23 \pm 1 $^{\circ}$ C ambient temperature, 12 -h dark/light cycle, and 55–56 % relative humidity). During the study, mice had *ad libitum* access to chow pellets and fresh water. After one week of acclimatization, formalin and writhing tests were used to assess the anti-nociceptive effect of betaine. For this purpose, animals were divided randomly into two categories (192 mice for the formalin test and 192 for the writhing test). In each category, seven experiments were designed with 4 groups in each (n = 6 in each group). The experimental procedure is illustrated by a flow chart (Fig. 1). Experimental procedures were done according to the guide for the care and use of laboratory animals to investigate experimental pain in animals [18,19], approved by the ethical committee of the Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran (1876, 2019.02.13). To avoid the possible effects of the animals' circadian rhythm, all experiments were done from 8 am to 2 pm. All experiments were performed blind to experimental condition.

2.2. Drugs

Betaine monohydrate, Morphine, Naloxone, Atropine, Chlorpheniramine, Cimetidine, Flumazenil and Cyproheptadine were purchased from Sigma (St. Louis, MO, USA). Also, ethanol and formalin were purchased from Merck (Darmstadt, Germany). For writhing tests, betaine was dissolved in ethanol (Merck, Darmstadt, Germany) so that the final concentration of ethanol was 1.6 %, whereas other drugs were dissolved in saline. In the formalin test, betaine and all other drugs were dissolved in saline. All drugs were injected intraperitoneally (i.p.) and the injection volume was 0.5 mL [20].

2.3. Formalin test

In experiment 1, animals were injected (i.p) with saline, betaine (10 mg/kg, 20, mg/kg, 30 mg/kg), morphine (5 mg/kg), and 30 min later received 10 μ L of the 1% formalin solution into the plantar surface of the right paw. The test was performed according to Hunskaar and Hole [21]. To minimize the possible effect of stress during the study, mice were placed inside a Plexiglas observation chamber (30 \times 30 \times 25 cm³) equipped with a mirror angled at 45 $^{\circ}$ below the chamber for 30 min per day for 3 days [22]. During the test, 30 min adaptation periods were applied to the animals, then the test was performed. Briefly, 30 min after formalin injection into the subplantar space of the right hind paw, the time spent for paw licking was determined 0–5 min (first phase) and 15–45 min (second phase) [23]. In experiment 2, animals were injected (i.p) with saline, betaine (30 mg/kg), naloxone (2 mg/kg) and betaine (30 mg/kg) + naloxone (2 mg/kg). In the co-injection group, first, the mice were pretreated with the antagonist and 15 min later received betaine (10 mg/kg) followed by formalin (10 μ L of the 1% solution) after 15 min. Then, the time spent for paw licking was determined in the first and second phase after formalin injection. In experiment 3, mice were injected (i.p) with saline, betaine (30 mg/kg), atropine (1 mg/kg) and a mixture of betaine (30 mg/kg) + atropine (1 mg/kg). Then, the time spent for paw licking was determined in the first and second phase after formalin injection. In experiment 4, injections consisted of saline, betaine (30 mg/kg), chlorpheniramine (20 mg/kg) and a mixture of betaine (30 mg/kg) + chlorpheniramine (20 mg/kg). Then, the time spent for the paw licking was determined in first and second phase after formalin injection. In experiment 5, animals were injected (i.p) with saline, betaine (30 mg/kg), flumazenil (5 mg/kg) and a mixture of betaine (30 mg/kg) + flumazenil (5 mg/kg). Then, the time spent for paw licking was determined in the first and second phase after formalin injection. In experiment 6, the injections were comprised of saline, betaine (30 mg/kg), cimetidine (12.5 mg/kg) and a mixture of betaine (30 mg/kg) + cimetidine (12.5 mg/kg). Then, the time spent for paw licking was determined in the first and second phase after formalin injection. In experiment 7, mice received i.p. injections of saline, betaine (30 mg/kg), cyproheptadine (4 mg/kg) and betaine (30 mg/kg) + cyproheptadine (4 mg/kg). Then, the time spent for paw licking was determined in the first and second phase after formalin injection. The doses of the drugs used were chosen based on the literature review and preliminary pilot study [2,9].

2.4. Writhing test

In the first experiment, mice were injected (i.p) with ethanol (10 mL/kg), betaine (10 mg/kg, 20, mg/kg, and 30 mg/kg), and morphine (5 mg/kg). Thirty minutes later, animals were injected (i.p) with 10 mL/kg of 0.6 % acetic acid and writhing test was done until 30 min post injection. Anti-nociceptive activity was determined as the percentage of inhibition of writhing based on the ratio of the percentage of inhibition: (control mean-treated mean) / (control mean) \times 100 % [2]. The acetic acid induced writhing test or abdominal contractions are an excessive extension of the abdominal region in combination with the extension of the hind limbs [24]. Then, the most effective level of betaine compared to the morphine was chosen for the rest of the experiments. In experiment 2, mice were injected (i.p) with ethanol (10 mL/kg), betaine (30 mg/kg), naloxone (2 mg/kg) and a mixture of betaine (30 mg/kg) + naloxone (2 mg/kg). In the co-injection group, first, the mice were pretreated with the antagonist. 15 min later betaine (10 mg/kg) was administered, and 30 min later, the writhing test was done and the inhibition percentage was calculated. In experiment 3, injections included ethanol (10 mL/kg), betaine (30 mg/kg), atropine (1 mg/kg) and a mixture of betaine (30 mg/kg) + atropine (1 mg/kg). Then, 30 min later, the writhing test was done and the inhibition percentage was calculated. In experiment 4, mice were injected (i.p) with ethanol (10 mL/kg), betaine (30 mg/kg), chlorpheniramine (20 mg/kg) and a

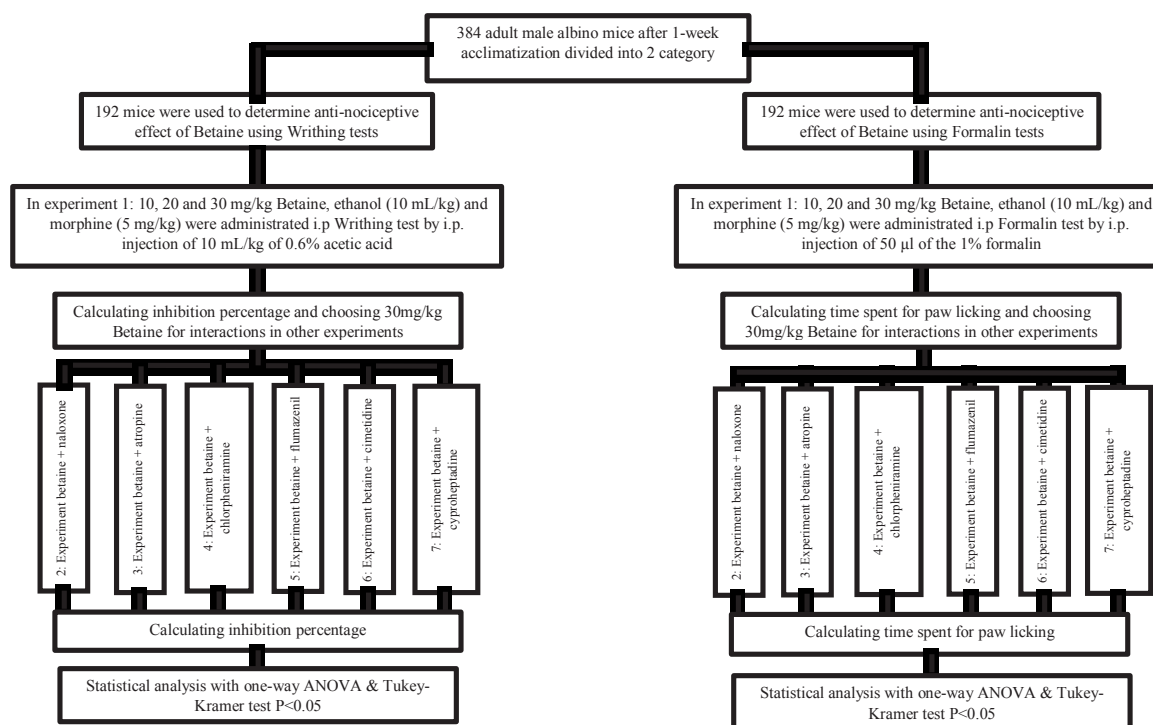


Fig. 1. Experimental procedure.

mixture of betaine (30 mg/kg) + chlorpheniramine (20 mg/kg). Then, 30 min later, the writhing test was done and the inhibition percentage was calculated. In experiment 5, animals were injected (i.p) with ethanol (10 mL/kg), betaine (30 mg/kg), flumazenil (5 mg/kg) and betaine (30 mg/kg) + flumazenil (5 mg/kg). Then, 30 min later, the writhing test was done and the inhibition percentage was calculated. In experiment 6, injections included ethanol (10 mL/kg), betaine (30 mg/kg), cimetidine (12.5 mg/kg) and betaine (30 mg/kg) + cimetidine (12.5 mg/kg). Then, 30 min later, the writhing test was done and the inhibition percentage was calculated. In experiment 7, mice received i.p. injections of ethanol (10 mL/kg), betaine (30 mg/kg), cyproheptadine (4 mg/kg) and betaine (30 mg/kg) + cyproheptadine (4 mg/kg). Then, 30 min later, the writhing test was done and the inhibition percentage was calculated.

2.5. Antioxidant activity

At the end of the study, blood samples were obtained from formalin-injected mice to determine superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GPx) levels. A thio-barbituric acid reaction-based method was used for assessment of MDA levels [25] by measuring absorption at 532 nm and expressed as nmol/g [26]. Glutathione Peroxidase (GPx) catalyzes the oxidation of glutathione and in the presence of glutathione reductase and NADPH, oxidized glutathione is converted to the reduced form by changes in oxidation of NADPH to NADP⁺. The GPx level was measured by the absorbance at 340 nm and expressed as U/mg [27]. The SOD activity was measured according to the method of Paoletti and Mocali, [28]. In brief, the superoxide anions were generated from manganese (II) chloride and mercaptoethanol in the presence of acid-ethylenediaminetetraacetic acid. The SOD level was determined on the basis of its ability to inhibit nicotinamide adenine dinucleotide (NAD) oxidation in a reaction mixture after the addition of serum. NAD oxidation was measured at 340 nm and expressed as U/mg. At the end of the study animals were sacrificed by euthanization with an overdose injection of pentobarbital (300 mg/kg, i.p.).

2.6. Rotarod test

The effect of betaine on motor coordination was assessed using a rotarod apparatus [29]. The mice (n = 30) were pretreated with saline, 10, 20 and 30 mg/kg of betaine or morphine (5 mg/kg), 30 min before testing on a rotarod [29]. During the training period, the mice were first trained on the rotarod at a constant speed of 20 rotations per minute (rpm) until all of the mice were able to spend at least 3 min on the rod [30]. The animals were selected 24 h previously and those which did not remain on the bar for two consecutive periods of 60 seconds were not employed in the test. Thirty minutes after injection of betaine or saline, mice were tested at 20 rpm for 10 min at 5-minute intervals for 30 min [9].

2.7. Statistical analyses

Data was analyzed by one-way analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA) and presented as mean ± SEM (standard error of the mean). For treatments showing a main effect by ANOVA the analysis was followed by a Tukey post-hoc test.

3. Results

3.1. Formalin test

The anti-nociceptive effects of betaine using the formalin test are presented in Fig. 2. As can be seen, betaine reduced pain in comparison to the control group (P = 0.001) in a dose-dependent manner. Betaine (30 mg/kg) induced a significant reduction in the licking and biting time of the injected paw compared to the control group in phases I and II (P = 0.001). Naloxone (2 mg/kg) had no significant anti-nociceptive effect using the formalin test in phases I and II (P = 0.453). Co-administration of the naloxone and betaine significantly decreased licking and biting time of the injected paw compared to the control group (P = 0.045). Co-administration of the naloxone + betaine significantly increased licking and biting time of the injected paw in comparison to the

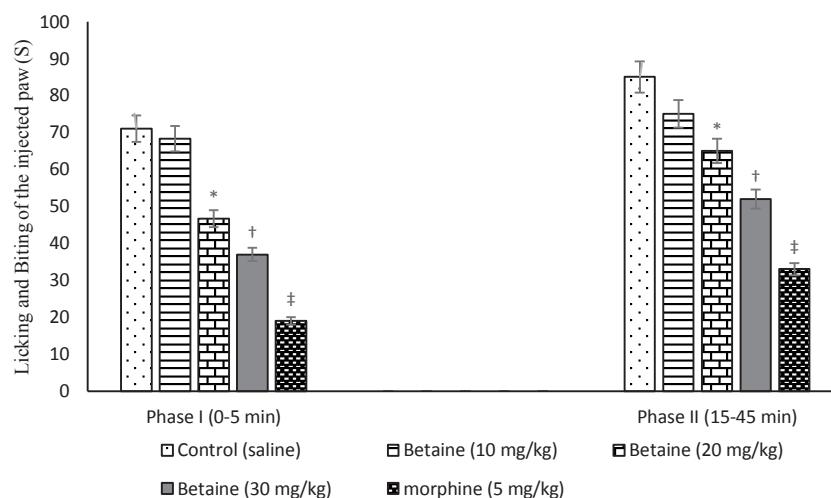


Fig. 2. Effect of the betaine on licking and biting of the injected paw in male mice (n = 30). Data are expressed as mean \pm SEM. *, †, ‡ P < 0.05 compared with control.

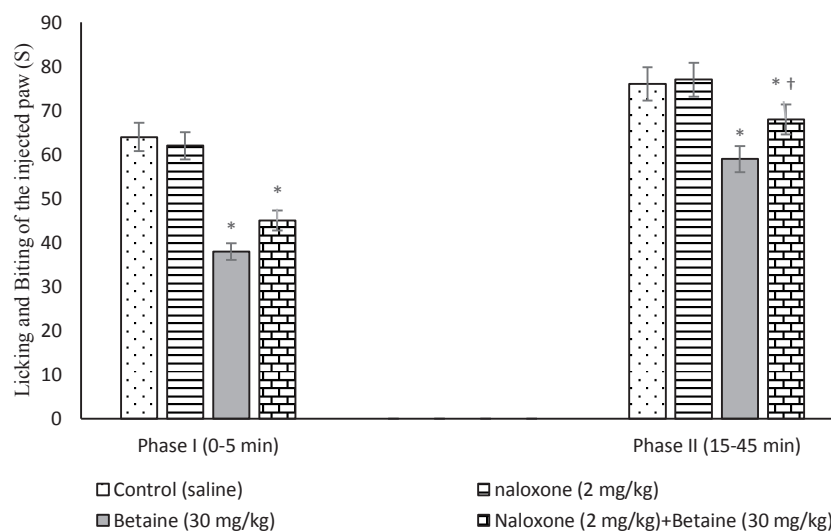


Fig. 3. The effect of betaine, naloxone and their co-injection on licking and biting of the injected paw in male mice (n = 30). Naloxone: opioid receptor antagonist, Data are expressed as mean \pm SEM. * P < 0.05 compared with control; † P < 0.001 naloxone + betaine compared with betaine.

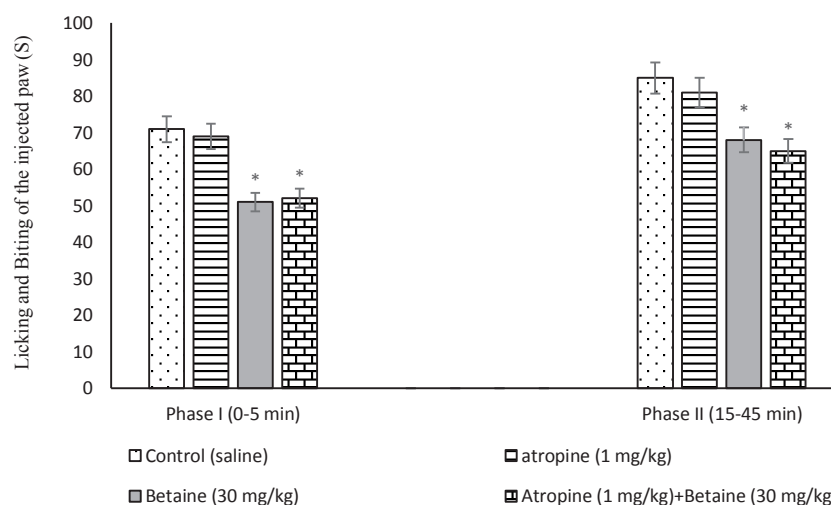


Fig. 4. The effect of betaine, atropine and their co-injection on licking and biting of the injected paw in male mice (n = 30). Atropine: muscarinic receptors antagonist. Data are expressed as mean \pm SEM. * P < 0.05 compared with control.

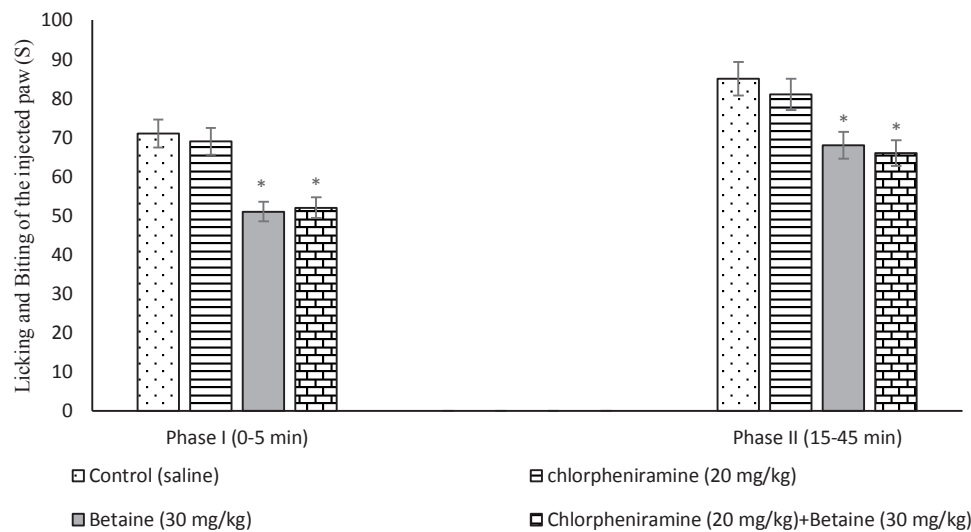


Fig. 5. The effect of betaine, chlorpheniramine and their co-injection on licking and biting of the injected paw in male mice ($n = 30$). Chlorpheniramine: histamine H_1 -receptor antagonist. Data are expressed as mean \pm SEM. * $P < 0.05$ compared with control.

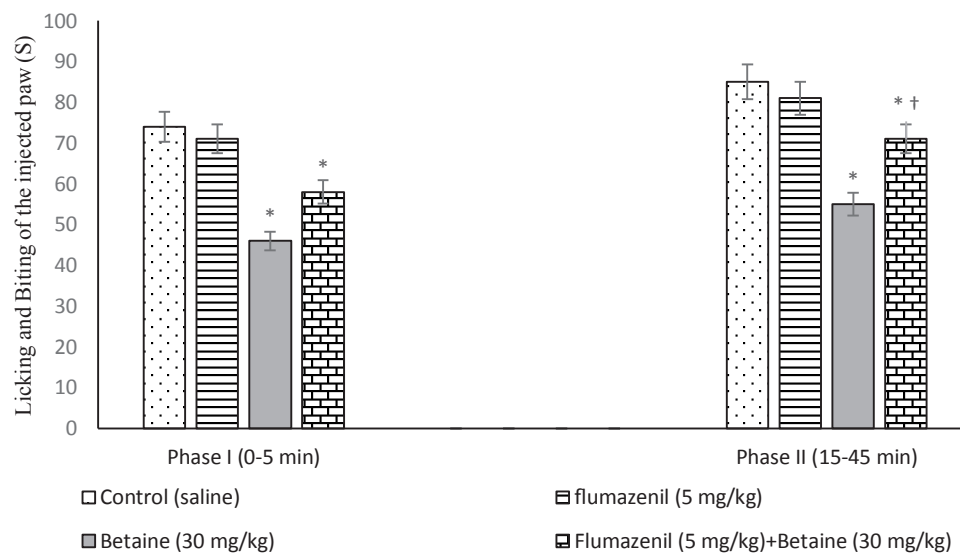


Fig. 6. The effect of betaine, flumazenil and their co-injection on licking and biting of the injected paw in male mice ($n = 30$). Flumazenil: selective $GABA_A$ antagonist. Data are expressed as mean \pm SEM. * $P < 0.05$ compared with control; † $P < 0.001$ flumazenil + betaine compared with betaine.

betaine group in phase II ($P = 0.047$; Fig. 3). These results suggest that blockage of opioid receptors using naloxone had an effect on the anti-nociceptive effects of betaine. It is possible that, the anti-nociceptive response of betaine is mediated via opioid receptors. The role of atropine on the anti-nociceptive effects of betaine is presented in Fig. 4. Administration of betaine (30 mg/kg) significantly diminished the licking and biting time of the injected paw compared to the control group in phases I and II ($P = 0.001$). Atropine (1 mg/kg) had no anti-nociceptive effect in phases I and II ($P = 0.451$). Co-administration of atropine + betaine had no significant effect on the anti-nociception effect of the betaine as seen by the licking and biting time of the injected paw in phases I and II ($P = 0.231$; Fig. 4). Betaine (30 mg/kg) significantly decreased the licking and biting time of the injected paw ($P = 0.001$). Chlorpheniramine (20 mg/kg) did not produce any anti-nociceptive response ($P = 0.540$). Co-administration of chlorpheniramine + betaine had no significant effect on the anti-nociception effect of the betaine as seen by the licking and biting time of the injected paw in phase I and II ($P > 0.05$; Fig. 5). As seen in Fig. 6, injection of betaine (30 mg/kg), significantly decreased the licking and biting time of the injected paw in phases I and II ($P = 0.001$). Flumazenil (5 mg/kg) had

no significant effect on the anti-nociceptive effect in phases I and II ($P = 0.423$) while co-administration of flumazenil + betaine, significantly diminished the anti-nociceptive effect of betaine on the licking and biting time of the injected paw compared to the control group in phase II ($P = 0.0312$). These results suggest that blockage of the GABA receptor using the flumazenil had an effect on the anti-nociceptive effects of betaine. It seems that the anti-nociceptive response of betaine is mediated via GABA receptors. Betaine (30 mg/kg) significantly reduced the licking and biting time of the injected paw compared to the control group in phases I and II ($P = 0.001$). Cimetidine (12.5 mg/kg) had no significant effect on anti-nociception in phases I and II ($P = 0.241$; Fig. 7). Co-administration of cimetidine + betaine had no significant effect on the anti-nociceptive effect of betaine on the licking and biting time of the injected paw in phases I and II ($P = 0.51$; Fig. 7). Based on the results of Fig. 8, betaine (30 mg/kg) diminished the licking and biting time of the injected paw in phases I and II ($P = 0.001$). Cyproheptadine (4 mg/kg) had no significant effect on anti-nociception in phases I and II ($P = 0.239$; Fig. 8). Co-administration of cyproheptadine + betaine had no significant effect on the anti-nociceptive effect of betaine on the licking and biting time of the

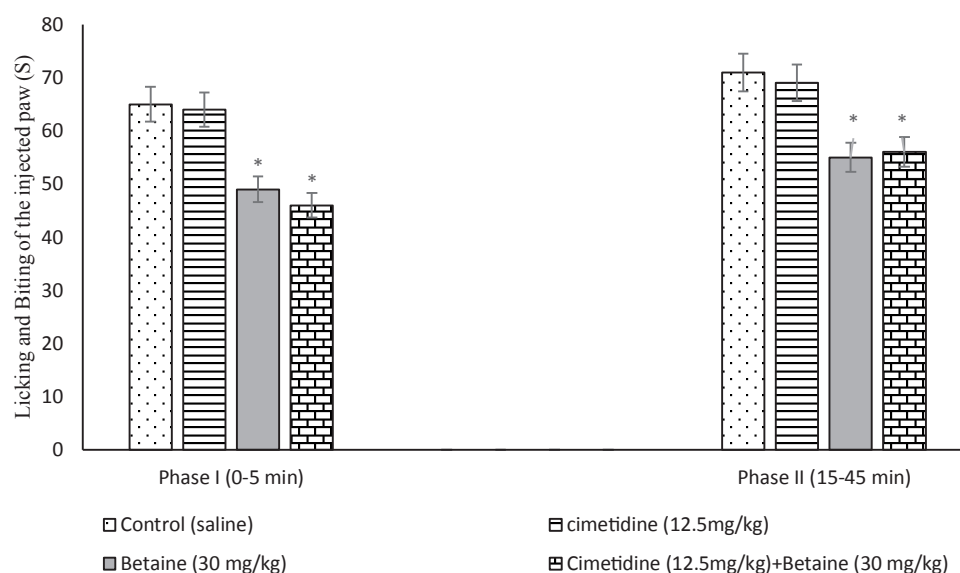


Fig. 7. The effect of betaine, cimetidine and their co-injection on licking and biting of the injected paw in male mice ($n = 30$). Cimetidine: histamine H_2 -receptor antagonist. Data are expressed as mean \pm SEM. * $P < 0.05$ compared with control.

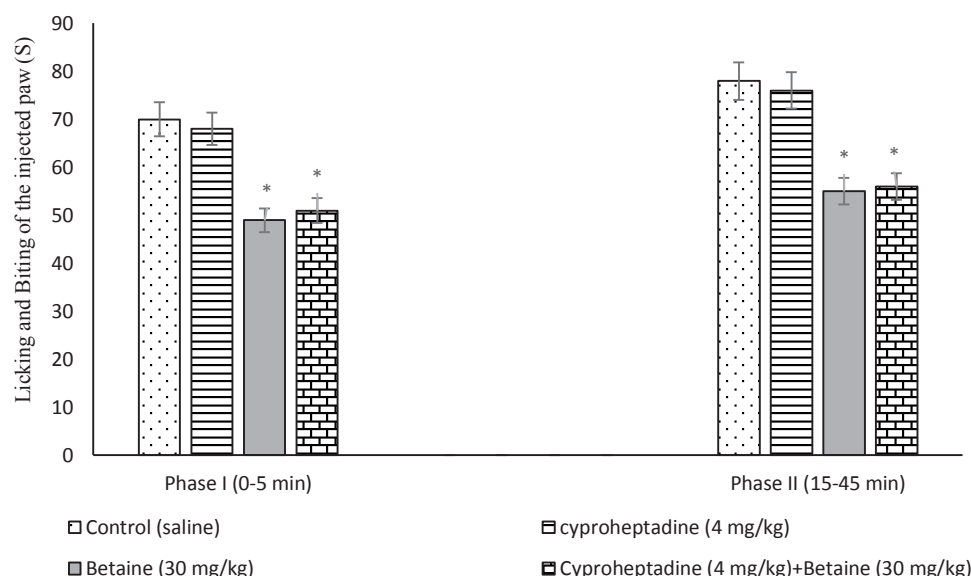


Fig. 8. The effect of betaine, cyproheptadine and their co-injection on licking and biting of the injected paw in male mice ($n = 30$). Cyproheptadine: serotonergic receptor antagonist. Data are expressed as mean \pm SEM. * $P < 0.05$ compared with control.

injected paw in phases I and II ($P = 0.624$).

3.2. Writhing test

Anti-nociceptive effects of the betaine writhing test is presented in Table 1A. As can be seen, betaine reduced pain in comparison to the control group ($P = 0.001$) in a dose-dependent manner. Also, morphine

Table 1A

. Effect of the different levels of the Betaine on Acetic Acid-Induced Writhing Test in Mice.

Experimental groups ($n = 6$)	Writhing count	Inhibition (%)	P value
Ethanol (10 mL/kg)	74.26 \pm 1.36	0	0.689
Betaine (10 mg/kg)	65.69 \pm 1.87	31.73 *	0.038
Betaine (20 mg/kg)	54.23 \pm 1.37	40.43 *	0.023
Betaine (30 mg/kg)	30.17 \pm 1.55	59.37 *	< 0.001
Morphine (5 mg/kg)	23.42 \pm 2.16	68.46 †	< 0.001

significantly decreased the number of writhing movements ($P = 0.001$). Betaine (30 mg/kg) significantly decreased inhibition in the pain response of 57.54 % compared to the control group ($P = 0.032$). Naloxone (2 mg/kg) had no significant anti-nociceptive response using the writhing test ($P = 0.453$). Co-administration of naloxone + betaine significantly decreased the number of writhing movements (47.48 %) compared to the control group ($P = 0.001$). Co-administration of naloxone + betaine significantly increased the number of writhing movements (47.48 %) in comparison to the betaine group (57.54 %) ($P = 0.041$; Table 1B). These results suggest that blockage of the opioid receptor using naloxone influenced the anti-nociceptive effects of betaine. It could be possible that the anti-nociceptive response of betaine is mediated via opioid receptors. Betaine (30 mg/kg) significantly diminished the pain response of 57.54 % compared to the control group ($P = 0.032$; Table 2A). Atropine (1 mg/kg) had no anti-nociceptive response ($P = 0.451$). Co-administration of atropine + betaine had no significant effect on the anti-nociceptive effect of the betaine in

Table 1B

Effect of the Naloxone on Betaine anti-nociception on acetic acid-induced Writhing Test in Mice.

Experimental groups	Writhing count	Inhibition (%)	P value
Ethanol (10 mL/kg)	74.10 ± 1.05	0	0.736
Naloxone (2 mg/kg)	74.26 ± 1.33	0	0.541
Betaine (30 mg/kg)	31.22 ± 1.34	57.54 [‡]	0.032
Naloxone + Betaine	38.62 ± 1.41	47.48 [§]	0.001

Values are presented as mean ± standard error of mean Naloxone: opioid receptor antagonist. *P < 0.05 Betaine compared with ethanol. † P < 0.05 morphine compared with ethanol. ‡P < 0.05 Betaine compared with naloxone. § P < 0.05 Naloxone + Betaine compared with naloxone.

Table 2A

Effect of the atropine on Betaine anti-nociception on acetic acid-induced Writhing Test in Mice.

Experimental groups (n = 6)	Writhing count	Inhibition (%)	P value
Ethanol (10 mL/kg)	73.54 ± 1.14	0	0.761
Atropine (1 mg/kg)	72.17 ± 1.68	0	0.431
Betaine (30 mg/kg)	33.18 ± 1.14	54.88 *	0.032
Atropine + Betaine	32.52 ± 1.28	55.75	0.453

Table 2B

Effect of the chlorpheniramine on Betaine anti-nociception on acetic acid-induced Writhing Test in Mice.

Experimental groups	Writhing count	Inhibition (%)	P value
Ethanol (10 mL/kg)	72.66 ± 1.11	0	0.684
Chlorpheniramine (20 mg/kg)	69.88 ± 1.94	0	0.267
Betaine (30 mg/kg)	31.49 ± 1.62	57.17 [†]	0.032
Chlorpheniramine + Betaine	30.17 ± 1.55	52.17	0.537

Values are presented as mean ± standard error of mean Atropine: muscarinic receptors antagonist. Chlorpheniramine: histamine H₁-receptor antagonist. *P < 0.05 betaine compared with Atropine. † P < 0.05 betaine compared with Chlorpheniramine.

Table 3A

Effect of the flumazenil on Betaine anti-nociception on acetic acid-induced Writhing Test in Mice.

Experimental groups (n = 6)	Writhing count	Inhibition (%)	P value
Ethanol (10 mL/kg)	73.20 ± 1.09	0	0.741
Flumazenil (5 mg/kg)	71.95 ± 1.22	0	0.364
Betaine (30 mg/kg)	32.04 ± 1.51	56.43 *	0.032
Flumazenil + Betaine	45.21 ± 1.10	38.52 [†]	< 0.001

reducing the number of writhing movements (55.75 %; Table 2B) (P = 0.453). Betaine (30 mg/kg) significantly decreased the pain response of 57.54 % in comparison to the control group (P = 0.032). Chlorpheniramine (20 mg/kg) did not produce any anti-nociceptive response (P = 0.540). Co-administration of chlorpheniramine + betaine had no significant effect on inhibiting the number of writhing movements (52.17 %; Table 3A) (P = 0.537).

As seen in Table 3B, injection of betaine (30 mg/kg) significantly decreased the pain response of 57.54 % compared to the control group

Table 3B

Effect of the Naloxone on cimetidine anti-nociception on acetic acid-induced Writhing Test in Mice.

Experimental groups	Writhing count	Inhibition (%)	P value
Ethanol (10 mL/kg)	71.33 ± 1.62	0	0.635
Cimetidine (12.5 mg/kg)	69.71 ± 1.48	0	0.453
Betaine (30 mg/kg)	33.14 ± 1.17	54.93 ^{††}	0.032
Cimetidine + Betaine	34.44 ± 1.87	53.16	0.541

Values are presented as mean ± standard error of mean. Flumazenil: selective GABA_A antagonist. Cimetidine: histamine H₂-receptor antagonist. *P < 0.05 betaine compared with Flumazenil. † P < 0.05 flumazenil + betaine compared with flumazenil. †† P < 0.05 betaine compared with Cimetidine.

Table 4

Effect of the cyproheptadine on Betaine anti-nociception on acetic acid-induced Writhing Test in Mice.

Experimental groups	Writhing count	Inhibition (%)	P value
Ethanol (10 mL/kg)	74.13 ± 1.84	0	0.462
Cyproheptadine (4 mg/kg)	65.69 ± 1.87	0	0.416
Betaine (30 mg/kg)	31.86 ± 1.14	56.67 *	0.032
Cyproheptadine + Betaine	33.44 ± 1.77	54.52	0.624

Values are presented as mean ± standard error of mean. Cyproheptadine: serotonergic receptor antagonist. *P < 0.05 betaine compared with Cyproheptadine.

(P = 0.032). Flumazenil (5 mg/kg) had no significant effect on anti-nociception (P = 0.423). Co-administration of flumazenil + betaine significantly decreased the number of writhing movements (38.52 %) compared to the control group (P = 0.001). These results suggest that blockage of the GABA receptor using flumazenil had an effect on the anti-nociceptive effects of betaine. It seems that the anti-nociceptive response of betaine is mediated via GABA receptors. Betaine (30 mg/kg) induced a significant inhibition in the pain response of 57.54 % of mice compared to the control group (P = 0.032). Cimetidine (12.5 mg/kg) had no significant effect on anti-nociception (P = 0.241 Table 4). Co-administration of cimetidine + betaine, had no significant effect on inhibiting the number of writhing movements (53.16 %; Table 3B) (P = 0.541). Based on the results of Table 4, betaine (30 mg/kg) diminished the pain response of 57.54 % of mice compared to the control group (P = 0.032). Cyproheptadine (4 mg/kg) had no significant effect on anti-nociception (P = 0.239; table). Co-administration of cyproheptadine + betaine had no significant effect on the anti-nociceptive effect of betaine on inhibiting the number of writhing movements (54.52 %; Table 4) (P = 0.624).

3.3. Anti-oxidant test

The anti-oxidant effect of betaine is presented in Table 5. As seen, betaine decreased the formalin-induced elevated MDA levels in comparison to the control group (P = 0.023) in a dose dependent manner. Also, betaine (10, 20 and 30 mg/kg) increased SOD and GPx levels in formalin-received mice in comparison to the control group (P = 0.042).

3.4. Rotarod activity test

Effects of betaine on locomotion and sedation are shown in Fig. 9. As can be seen, no significant effect was observed on locomotion and sedation in betaine (10, 20 and 30 mg/kg) treated mice compared to morphine (5 mg/kg) (P = 0.634).

4. Discussion

Based on the literature and our knowledge, this is the first report on the anti-nociceptive effect of Betaine using both formalin and writhing-induced pain tests as well as its antioxidant activity following

Table 5

Effect of different levels betaine on serum values of Malondialdehyde, Superoxide dismutase and Glutathione peroxidase followed by formalin injection in mice.

Group	MDA (nmol/g)	SOD (U/mg)	GPx (U/mg)
Control (formalin)	162.11 ± 6.14	2.11 ± 0.12	2.27 ± 0.26
Betaine (10 mg/kg)	156.13 ± 7.21 *	2.87 ± 0.14 *	2.86 ± 0.34 *
Betaine (20 mg/kg)	140.10 ± 7.55 *	3.01 ± 0.13 *	3.12 ± 0.18 *
Betaine (30 mg/kg)	124.26 ± 7.23 [†]	4.11 ± 0.10 [†]	4.26 ± 0.41 [†]

MDA: malondialdehyde, SOD: superoxide dismutase, GPx: glutathione peroxidase. *† P < 0.05, 0.001 betaine compared with control.

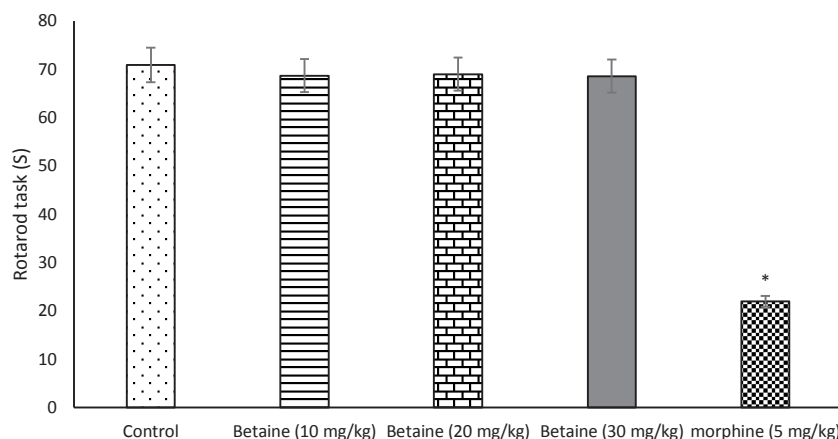


Fig. 9. Effect of the betaine on in the rotarod task in mice ($n = 30$). Data are expressed as mean \pm SEM. * $P < 0.05$ compared with control.

inflammatory pain. Based on the primary outcome of the paper, the results showed that betaine had a dose dependent anti-nociceptive response. The formalin and acetic acid writhing tests are animal model inflammatory pain experiments created to determine the analgesic/anti-inflammatory potency of different compounds [31]. Injection of the plantar formalin or intraperitoneal acetic acid induces chemical leads via release of pro-inflammatory mediators, activates peripheral nociceptors on the sensory nerve fibers and leads to pain and hyperalgesia [32]. Also, morphine (5 mg/kg) as a reference drug, significantly decreased the licking and biting time of the injected paw and the number of writhing movements using formalin and writhing tests, respectively. The dose of the morphine used was based on previous reports [33]. Two phases of pain were evoked by formalin injection into the hind paw of rodents: phase I consists of neurogenic nociception by direct stimulation of nociceptors via C fibers to the dorsal horn of the spinal cord after substance P is secreted and acts as a neurotransmitter. The second phase consists of inflammatory-induced pain due to the release of serotonin, histamine, bradykinin and prostaglandins (PGs) from the tissue that is damaged by formalin [33].

Based on the objective of the current study, we investigated the interaction of its anti-nociceptive activity with opioidergic, histaminergic, muscarinic, GABAergic and serotonergic receptors in formalin and writhing-induced pain. Co-administration of naloxone + betaine significantly increased the licking and biting time of the injected paw in comparison to the betaine group in phase II (Fig. 3A). Also, co-administration of naloxone + betaine significantly decreased the number of writhing movements (47.48 %) compared to the control group. Co-administration of naloxone + betaine significantly increased the number of writhing movements (47.48 %) in comparison to the betaine group (57.54 %) (Table 1B). Naloxone is a nonselective antagonist for μ , δ and κ receptors and because naloxone administration inhibits the anti-nociceptive effects of betaine, it seems that the anti-nociceptive response of betaine is mediated via opioid receptors. Opioid receptors μ , κ and δ are identified in the CNS and mediated to the peripheral tissues in pain and analgesia, tolerance and dependence [34]. Naloxone is widely used to investigate the role of the endogenous opioid analgesic system in pain modulation [35]. Naloxone is a competitive antagonist of the μ , κ , and δ receptors, with a high affinity for the μ opioid receptors [36]. Betaine is a methyl donor in the betaine homocysteine methyltransferase pathway and leads to homocysteine reduction and increased blood S-adenosylmethionine levels [37]. It is reported that betaine has the potential to treat neurological disorders such as seizures and Rett syndrome [9]. Betaine attenuates memory deficits induced by homocysteine [38] and lipopolysaccharide [12]. It is assumed that betaine attenuates memory deficits and has an important role in the regulation of brain functions in forced swimming test (FST), novelty suppressed feeding test (NSF), prepulse inhibition test,

Novelty suppressed feeding test, novel object recognition test and open field test. [9].

As can be seen, co-administration of flumazenil + betaine significantly increased licking and biting time of the injected paw in comparison to the betaine group in phase II (Fig. 4B). Additionally, co-administration of flumazenil + betaine significantly decreased the number of writhing movements (38.52 %) compared to the control group. Co-administration of flumazenil + betaine significantly increased the number of writhing movements (38.52 %) in comparison to the betaine group (56.43 %) in phase II (table 3B). Betaine/GABA transporter (BGT1), the mouse transporter homologue of which is known as GABA transporter 2 (GAT2) has high homology to the GABA transporters (GAT1, GAT2 and GAT3) and is a Na^+ and Cl^- dependent neurotransmitter transporter gene family with a high homology to the GABA transporters (GAT1, GAT2 and GAT3) [38,39]. Since GABA is the major inhibitory neurotransmitter in the CNS, betaine and GABA transporters could be useful in the therapy of CNS disorders such as neuropathic pain [40]. BGT1 is localized in astrocytes distant from GABAergic synapses and perhaps has another role in GABAergic transmission from GABA transporters [41]. BGT-1 is capable of utilizing both betaine and GABA and its expression upregulates neuronal injury in the rat hippocampus. Betaine has protective effects against c-induced memory impairment and prevention of lipopolysaccharide-induced changes in GAT2 mRNA expression is crucial to this ameliorating effect [13].

In the limbic system, the rostral anterior cingulate cortex participates in the supraspinal generation of nociceptive signals from the periphery and GABA_A has a more prominent role [42,43]. Locally released GABA in the amygdala is related with collecting and processing pain information and exerting an inhibitory control mediated via GABA_A receptors which decrease neural excitability [43]. Both GABA_A and GABA_B receptors adjust pain communication at the dorsal horn established on the primary afferent terminals in addition to dorsal horn neurons [19]. Additionally, the GABAergic system interacts with opioidergic, muscarinic, cannabinoidergic and adenosine receptors in anti-nociception at the spinal and supraspinal levels [44]. Betaine attenuates glutamate-induced neurotoxicity in primary cultured brain cells. Based on the structural similarity to glycine, we examined if betaine can affect the NMDAR function and demonstrated that betaine acts like a NMDAR glycine binding site partial agonist [9].

Although betaine and GAT2/BGT-1 may be involved in neuronal dysfunction caused by neurodegeneration or neuronal injury, their physiological roles are not fully elicited. Based on the secondary purpose of the current paper, findings revealed that betaine had no sedative and locomotion effect on acetic acid treated mice. As observed, betaine decreased MDA levels while increasing SOD and GPx levels in formalin-received mice. Betaine prevents inflammatory processes

through suppression of the expression of pro-inflammatory genes such as NF- κ B [45]. It also suppresses ROS generation [44]. The mechanism of this oxidative stress returns to auto-oxidation and formation of intracellular SOD and GPx. Oxidative stress concomitant scavenging of ROS by betaine increase catalase (CAT) activity in betaine-treated rats [46]. Due to the antioxidant and methyl donor properties, betaine has an antioxidant effect against oxidative damage in the testis and increases sperm motility [46]. Betaine decreases GPx and CAT activities in betaine-treated rats suggesting that it has antioxidative effects and preserves cellular antioxidativity [46,47]. There are limited reports on the anti-inflammatory and anti-oxidative activity of betaine since dietary data were not available [48]. It is reported that AOM/DSS-induced IL-6, COX-2, TNF- α and iNOS were inhibited by betaine treatment in the colonic mucosa [17]. Additionally, betaine decreases ROS generation, restoring the redox balance via maintaining thiol homeostasis and NF- κ B activation in endothelial cells [48]. Betaine (0.163 mmol/kg) improved LPS-induced memory impairment in rat [13]. Betaine inhibits chronic ethanol-induced oxidative stress in the brain which is a useful compound for preventing neurodegenerative, inflammatory processes and oxidative stress [49]. Betaine attenuates glutamate-induced neurotoxicity in primary cultured brain cells [50]. This finding revealed that betaine has a neuroprotective effect during inflammatory processes by mediating antioxidant status [46] and inflammatory mediators [13]. Although the mechanism of betaine's biological effects remains unclear, it seems that homocysteine methyltransferase as the primary enzymatic product of betaine acts as a powerful antioxidant molecule [12]. However, in this regard, there is no report for how the antioxidant activity of betaine interacts with its analgesic effect. Therefore, the novel findings of the current study on the anti-nociceptive activity of betaine can be used as base information to determine the anti-nociceptive mechanism of action of betaine in future studies. In conclusion, taking into account the new findings of the current study, betaine has anti-nociceptive and antioxidant activity in mice and its anti-nociceptive role interacts with opioidergic and GABA receptors.

Authors statement

Experimental procedures approved by the ethical committee of veterinary Faculty, Science and Research Branch, Islamic Azad University, Tehran, Iran (1876, 2019.02.13). There is no human subjects in this study.

Declaration of Competing Interest

There is no conflict of interest.

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