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Implication of NMDA-NO pathway in the antidepressant-like effect of ellagic acid in male mice

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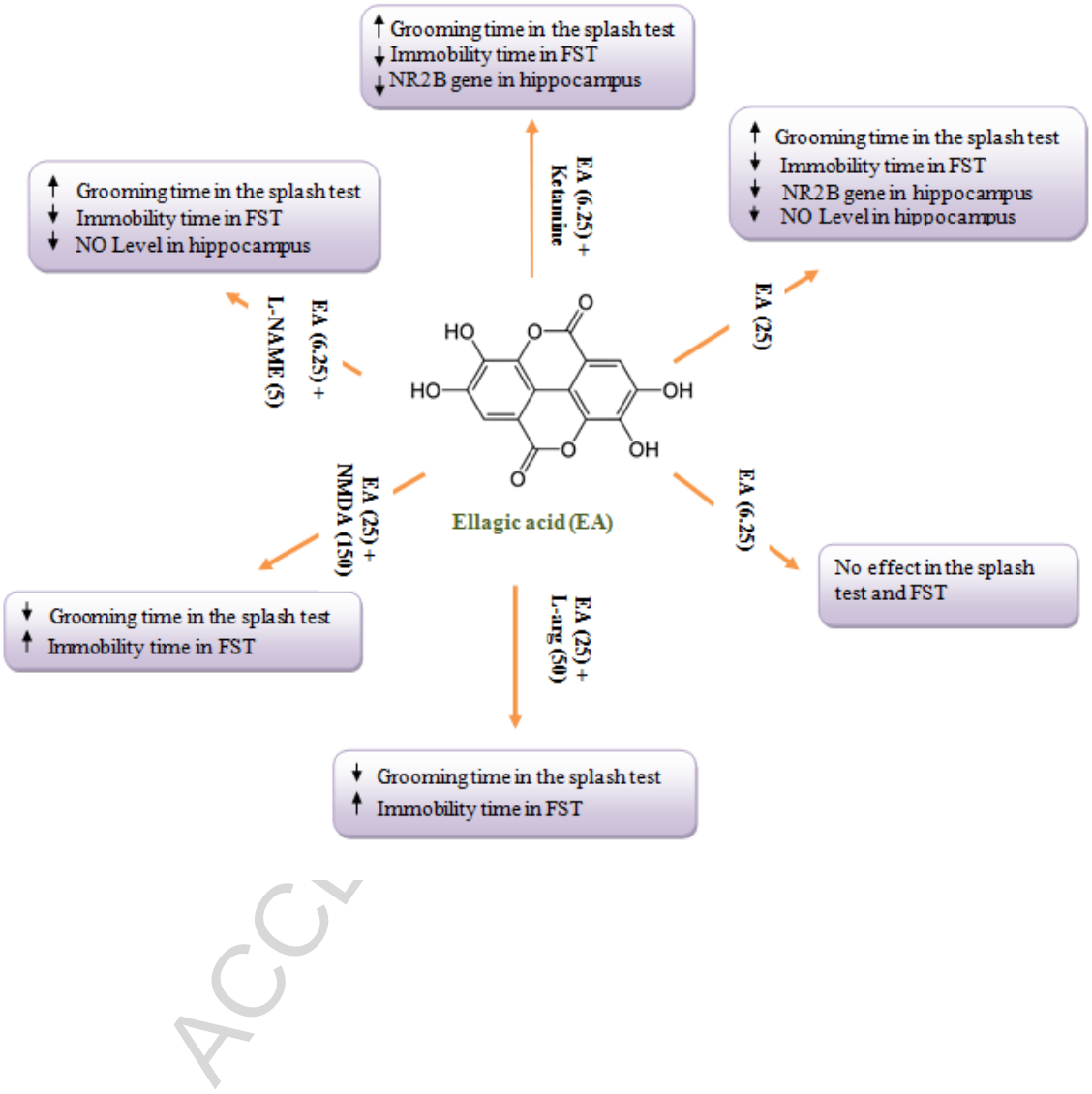
Abstract

Depression is one the common psychiatric disorders through the world. Nitric oxide (NO) and N-methyl-D-aspartate receptor (NMDA-R) are involved in the pathophysiology of depression. Previous studies have been reported various pharmacological properties for ellagic acid (EA). We aimed to evaluate possible involvement of NMDA-NO pathway in the antidepressant-like effect of EA. To do this, we used relevant behavioral tests to evaluate depressive-like behavior. In order to find effective and sub-effective doses of agents, mice treated with EA (6.25, 12.5, 25, 50 and 100 mg/kg), L-NAME (5 and 10 mg/kg), L-arg (25 and 50 mg/kg), NMDA (75 and 150 mg/kg) and ketamine (0.25 and 0.5 mg/kg). Furthermore, mice were treated with combination of sub-effective dose of EA plus sub-effective doses of L-NAME and/or ketamine as well as treated with effective dose of EA in combination of effective doses of L-arg and/or NMDA. Level of NO and gene expression of NR2A and NR2B subunits of NMDA-R were assessed in the hippocampus. Results showed that EA dose dependently provoked antidepressant-like effects and also decreased the hippocampal NO level as well as expression of NMDA-Rs. Co-administration of sub-effective doses of L-NAME or ketamine with sub-effective dose of EA potentiated the effect of EA on behaviors, NO level as well as NMDA-Rs gene expression in the hippocampus. However, co-treatment of effective dose of EA with effective doses of L-arg or NMDA mitigated effects of EA. In conclusion, our data suggested that NMDA-NO, partially at least, are involved in the antidepressant-like effect of EA.

Keywords: Ellagic acid; NMDA-NO pathway; antidepressant-like effect; mice

Graphical abstract



Abbreviations

EA: ellagic acid, NO: nitric oxide, NMDA-R: N-methyl-D-aspartate receptor, NOS: nitric oxide synthase, L-arg: L-arginine, L-NAME: NG-L-arginine methyl ester, OFT: open field test, FST: forced swimming test.

Introduction

Currently, depression is recognized as one of the most common psychiatric disorders throughout the world (Ghanean, Ceniti et al. 2018). Depression now has become a community health concern, because is associated with high morbidity and mortality; it brings a lot of economic burden to society (Silva, Galvao et al. 2014, McIntyre, Ng-Mak et al. 2017). Commonly prescribed antidepressants which are mostly regulating monoaminergic transmission exert few desired outcomes and many patients don't have complete response to these drugs (Arroll, Macgillivray et al. 2005, Berton and Nestler 2006). Considering the fact of increasing prevalence of depression (Compton, Conway et al. 2006), there is a pressing need to introduce new effective agents with promising pharmacological possessions and low side effects.

In this regard, several studies have been determined that glutamate, an excitatory amino acid, plays a potential role in the pathophysiology of mood disorders such as depression (Sanacora, Zarate et al. 2008, Ostadhadi, Ahangari et al. 2016). It has been shown that increase in the hippocampal glutamatergic activity *via* NMDA receptors change the hippocampus plasticity and participates in the pathophysiology of depression (Amiri, Alijanpour et al. 2016). Clinical evaluations revealed that the treatment with NMDA receptor antagonists such as ketamine mitigated the depressive symptoms in patients (Zarate, Singh et al. 2006). Also, preclinical studies showed that acute administration of NMDA antagonists produces antidepressant-like effects in rodents (Autry, Adachi et al. 2011, Ostadhadi, Khan et al. 2016, Lee, Ko et al. 2018). Evidences demonstrated that NMDA receptors are involved in the mechanism of action of certain antidepressant drugs (Szasz, Mike et al. 2007).

It has been well-determined that activation of NMDA receptors stimulates NO production in the brain. NO, synthesized from L-arginine by NOS, is a mediator in the CNS and it is involved in neurotransmission and synaptic plasticity (Esplugues 2002). It has been determined that NO is involved in regulation of several (patho) physiologic behavioral and emotional functions (Zhu, Dong et al. 2017, Inserra, Mastronardi et al. 2018, Matsumoto, Matsumoto et al. 2018). NO/cGMP signaling pathway has been known as an interesting target for mood disorders (yan Zhou, Zhang et al. 2017, Walia, Garg et al. 2018). Previous studies have demonstrated that NOS inhibitors possessed antidepressant-like effects in rodents as well as mediated antidepressant-like properties of effective agents (Tomaz, Cordeiro et al. 2014, Khan, Ostadhadi et al. 2016). In this regards, previous studies showed that inhibition of NMDA-NO pathway is involved in the antidepressant-like effects of some related agents (Contestabile 2000, Khan, Ostadhadi et al. 2016, Araújo, de Melo et al. 2018).

2,3,7,8 tetrahydroxybenzo pyrano [5, 4, 3-cde] benzopyran-5-10-dione, a polyphenolic metabolite, known as EA is present either in free form or as part of more complex molecules (ellagitannins). EA is found in many plants such as cranberries, pecans, pomegranate, grapes, raspberries seed, blackberries, strawberries and nuts (Ríos, Giner et al. 2018). Previous studies have been reported several pharmacological properties for EA including anti-bacterial, anti-inflammatory, immunoregulatory and anti-tumor effects (Chao, Hsu et al. 2009, García-Niño and Zazueta 2015, Ríos, Giner et al. 2018). It has been demonstrated that EA via attenuation of nitric oxide level exerts neuroprotective effect in the brain and sciatic nerve in streptozotocin-induced diabetic rats (Uzar, Alp et al. 2012). It has been suggested that EA exhibits neuro-protective effect and protects the brain from oxidative and inflammatory challenges (Uzar, Alp et al. 2012, Farbood, Sarkaki et al. 2015). Preclinical examinations determined antidepressant-like effects for EA (Dhingra and Chhillar 2012,

Girish, Raj et al. 2012), however, its mechanism of action has not yet been clearly specified and further studies are warranted to determine the mechanism of the antidepressant-like action of EA.

Considering the above-mentioned studies which suggested 1) a critical role for the NMDA-R/NO pathway in the pathogenesis of mood disorders such as depression and also 2) neuroprotective effect for EA, in the current study we aimed to evaluate the involvement of the NMDA-R/NO pathway in the antidepressant-like effect of EA in male mice.

Considering the above-mentioned studies which suggested a critical role for the NMDA-NO pathway in the pathogenesis of the depression and also with regard to the neuro-protective effects mentioned for the EA, the current study aimed to evaluate the involvement of the NMDA-NO pathway in the antidepressant-like effect of EA in male mice.

Material and methods

Animal

Three hundred and twelve male NMRI mice weighing 25-30 g were obtained from the Pasteur Institute, Tehran, Iran. Mice were kept at standard laboratory conditions including temperature of 21-23 ^{0}C, 12h regular light/dark cycle and free access to food and water. All tests were done between 10:00 and 14:00 h. All stages of experimentation were carried out in accordance with the regulations of the University and the Guide for the Care and Use of Laboratory Animals of National Institutes of Health (Ethics code: IR.SKUMS.REC.1397.18) and Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). Full efforts were made to diminish the use of animals and to improve their wellbeing. Animal randomly divided into the experimental groups (n=8 in each group). In order to prevent the effects of manipulation by different experimenters on animals and to

minimize manipulations, all experiments were conducted by a single experimenter. Each experimental group was contained 8 mice for behavioral tests and 4 samples for molecular assessments (gene expression and NO level).

Treatments

The following agents were used in this study: 1) EA. 2) L-NAME, a non-selective nitric oxide synthase or NOS inhibitor. 3) L-arg, a NO precursor. 4) NMDA, a selective agonist of the NMDA receptors. 5) Ketamine, a non-selective antagonist of the NMDA receptors. All drugs were bought from the Sigma, St Louis, MO, USA. All drugs were dissolved in physiological saline contained 5% DMSO (Haj-Mirzaian, Amini-Khoei et al. 2017) which considered as vehicle. All drugs were administrated *via* intraperitoneal (i.p.) rout with a volume of 5ml/kg body weight. We treated mice with L-arg (30min), L-NAME (45 min), NMDA (15 min), ketamine (60 min) and EA (60 min) prior to tests. The dose and time of drug injections were chosen according to previous studies (Girish, Raj et al. 2012, Amiri, Alijanpour et al. 2016, Amiri, Haj-Mirzaian et al. 2016, Bedel, Kencebay Manas et al. 2017, Haj-Mirzaian, Amiri et al. 2018), as well as our pilot studies. Each mouse was used only for one test (same mice were used for the FST and the OFT and different set of mice were used for the splash test).

Study design

In the current study we performed valid behavioral tests are relevant to depression including open field test (OFT), forced swimming test (FST) and splash test. Our study was consisted of 5 steps (Fig 1) as follows:

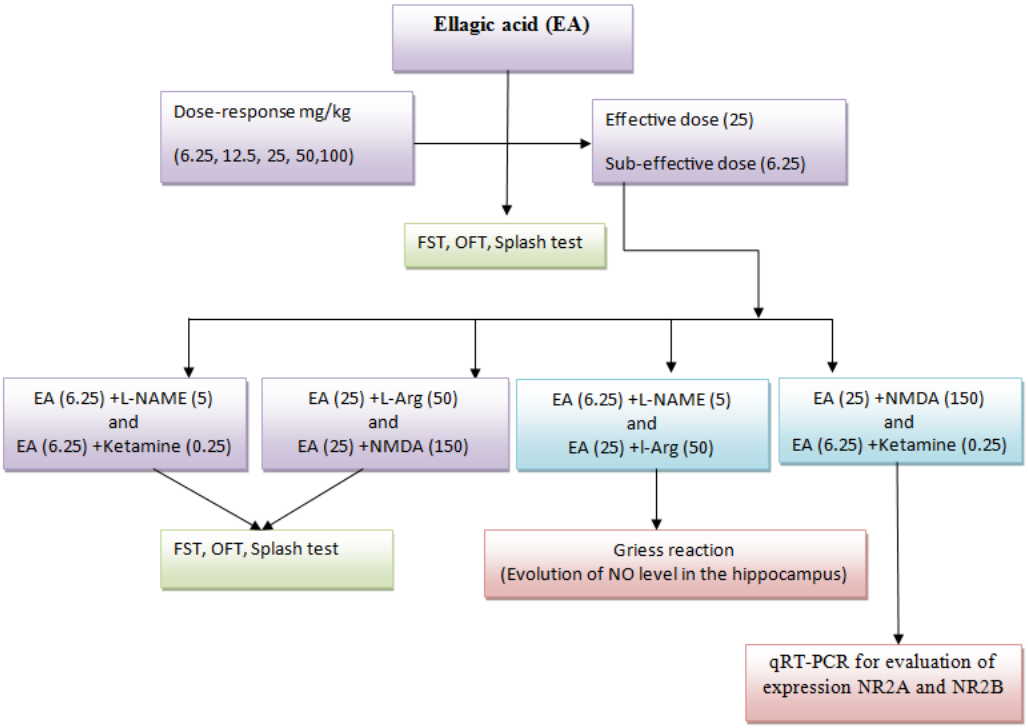


Fig 1. Schematic of the study design

Experiment 1: dose-effect study of EA

Examined the effects of different doses of the EA (6.25, 12.5, 25, 50 and 100 mg/kg, 60 min prior to tests) on the behavioral tests. The goal of this step was to find the effective and sub-effective doses of the EA.

Experiment 2: dose-effect study of NOS inhibitor/activator

Evaluated the effects of L-NAME (5 and 10 mg/kg, 45 min prior to tests) and L-arg (25 and 50 mg/kg, 30 min before tests) on depressive-like behaviors. In this step we aimed to find effective and sub-effective doses of the L-NAME and L-arg.

Experiment 3: dose-effect study of NMDA agonist/antagonist

We evaluated the effects of the NMDA (75 and 150 mg/kg, 15 min prior to tests) and the ketamine (0.25 an 0.5 mg/kg, 60 min before tests) on depressive-like behaviors. In this experiment we expected to find effective and sub-effective doses of the NMDA and ketamine.

Experiment 4: evaluation of possible involvement of NO in the effect of EA

We examined the effect of co-treatment of effective dose of the EA (25 mg/kg) with effective dose of the L-arg (50 mg/kg) on depressive-like behaviors. We also evaluated the effect of co-administration of sub-effective dose of EA (6.25 mg/kg) with sub-effective dose of L-NAME (5 mg/kg). Animals in this trial received L-arg 30 min prior to test and 30 min after EA as treated with L-NAME 45 min prior to test and 15 min after EA (Fig 2).

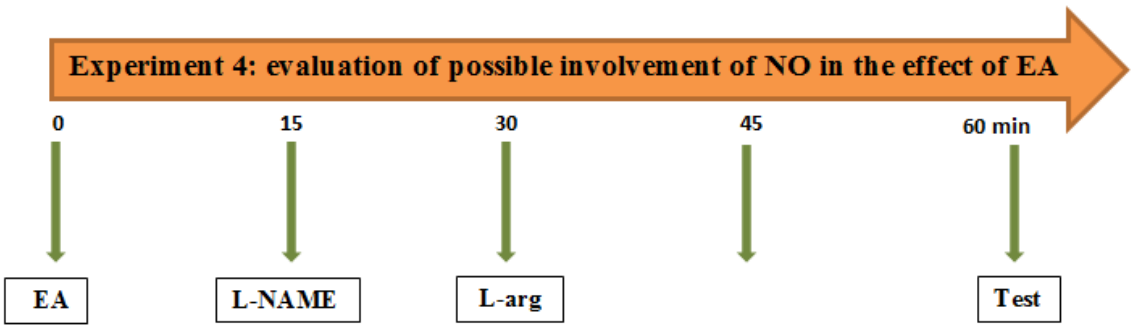


Fig 2. Schematic of the experiment 4

Experiment 5: evaluation of possible involvement of NMDA in the effect of EA

The effect of co-treatment of effective dose of the EA (25 mg/kg) with effective dose of the NMDA (150 mg/kg) on depressive-like behaviors was evaluated. Moreover, the effect of co-administration of sub-effective dose of EA (6.25 mg/kg) with sub-effective dose of ketamine (0.25 mg/kg) was assessed. Animals in this trial received NMDA 15 min prior to test and 45 min after EA as treated with ketamine and EA as simultaneously (60 min before test) (Fig 3).

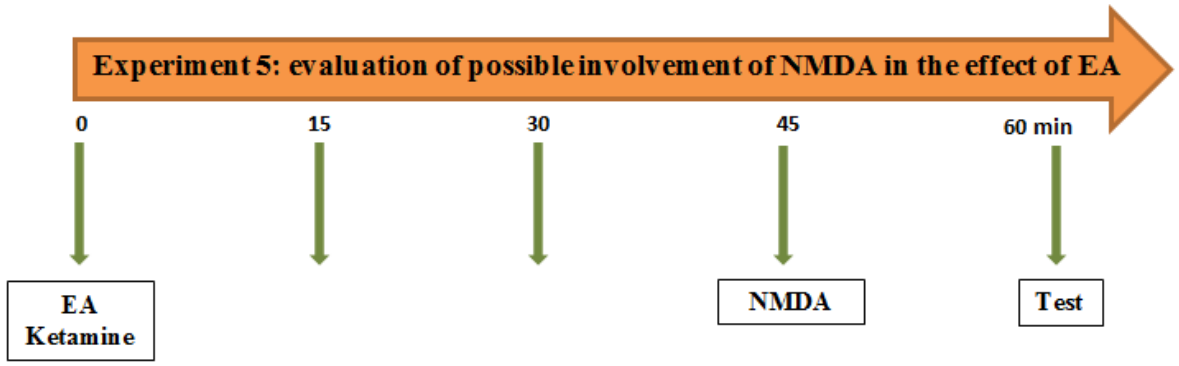


Fig 3. Schematic of the experiment 5

Behavioral tests

Forced swimming test (FST)

FST was performed as a valid test for measuring despair behavior in mice which observes as increased in the immobility time (Porsolt, Bertin et al. 1977, Lu, Ross et al. 2008) . In this test, mice were individually located in an open glass cylinder (diameter: 10 cm, height: 25 cm) occupied with 19 cm water (23 ± 1 °C). Animals were allowed to swim for duration of 6 min and the immobility time was recorded in the latter 4 min by an experimenter blinded to the treatments. Motionless time was considered when the mouse continued immobile in the water and did only those essential movements to keep its head above the water.

Open field test (OFT)

The OFT was performed to evaluate the locomotion following treatments (Amiri, Alijanpour et al. 2016). The OFT was done immediately before the FST to consider ambulatory behavior as well as to confirm that adjustments which occur in motor activity did not affect the immobility time in the FST(Rodrigues, Rocha et al. 1996) . The OFT device was made of white opaque Plexiglas

(50 cm × 50 cm × 30 cm), which was softly illumined. Each mouse was mildly situated on the center square (30 cm × 30 cm), and presentations were recorded by a camera for 5 min. The apparatus was cleaned with 70% ethanol after experiment of each mouse. The distance moved (horizontal activity, number of crossing by 4 foots from each square) and the number of rearings (vertical activity) was recorded.

Splash test

We performed the splash test to evaluate the self-care behavior. In this test grooming activity time designated an indirect ration for palatable solution drinking. To do this, a 10% sucrose solution was scattered on the dorsal coat of mice while they were in their home cages and mice were recorded for 5 min. Grooming movements were including nose/face cleaning, head washing, and body grooming which were recorded by a person blinded to treatments (Ducottet and Belzung 2004) .

Nitrite assay

Nitrite level was measured in the hippocampus samples based on method described in previous studies (Kordjazy, Haj-Mirzaian et al. 2015, Haj-Mirzaian, Amiri et al. 2018). In brief, mice were sacrificed under anesthesia using diethyl ether, and hippocampus was dissected on the ice-cold surface and directly place into liquid nitrogen. Hippocampus homogenates were prepared, and nitrite concentrations were measured using colorimetric assay based on the Griess reaction. Briefly, each well was loaded with 100 µL samples and mixed with 100 µL Griess reagent. The absorbance was measured at 540 nm in an automated plate reader, after ten minutes incubation at room temperature. The level of Nitrite was determined by reference to a standard curve of sodium nitrite (Sigma, USA) and normalized to the weight of each sample.

Real-time RT-PCR analysis for assessment of the NR2A and NR2B NMDA receptors genes expression

TRIzol reagent (Invitrogen) was used to extract total RNA from the hippocampi. After the reverse transcription of 1 μg of RNA using PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan), qRT-PCR was done to determine alterations in the mRNA levels of favorite genes. qRT-PCR was done on a light cycler device (Roche Diagnostics, Mannheim, Germany) using SYBR Premix Ex Taq technology (Takara Bio). Thermal cycling conditions were including an initial activation step for 30 s at 95 °C afterwards 45 cycles as well as a denaturation step for 5 s at 95 °C and a combined annealing/extension step for 20 s at 60 °C. Melting curve analysis was done to confirm whether all primers generated a single PCR product. The genes and their primers are listed in Table 1. Histone H2A variant, H2afz, was used as normalizer gene and variations in expression of each mRNA in comparison with H2afz was measured based on $2^{-\Delta\Delta Ct}$ relative expression formula, as described previously (Murgatroyd, Peña et al. 2015, Amiri, Haj-Mirzaian et al. 2017).

Table 1: primer sequences

name	sequence
Nr2a-F	CTCAGCATTGTCACCTTGGA
Nr2a-R	GCAGCACTTCTTCACATTCAT

Nr2b-F	CTACTGCTGGCTGCTGGTGA
Nr2b-R	GACTGGAGAATGGAGACGGCTA
H2afz-F	TCATCGACACCTGAAATCTAGGA
H2afz-R	AGGGGTGATACGCTTTACCTTTA

Statistics

Comparison between the groups was analyzed using one-way and two-way analyses of variance (ANOVAs), followed by tukey's multiple comparison test. GraphPad Prism software (version 7) was used for data analysis. $P < 0.05$ was considered statistically significant.

Results

Effect of EA **on the depressive-like behaviors**

One-way ANOVA revealed that there are significant difference among experimental groups in the FST ($F(5, 42) = 19.03$, $P<0.001$), splash test ($F(5, 42) = 25.47$, $P<0.001$), horizontal activity in the OFT ($F(5, 42) = 2.343$, $P<0.05$). However there is no significant difference among groups in the vertical activity of the OFT ($F(5, 42) = 1.388$, $P>0.05$). Tukey's post analysis showed that administration of EA at doses of 25 ($P<0.05$), 50 ($P<0.001$) and 100 mg/kg ($P<0.001$) significantly decreased the immobility time in the FST n the splash test our data exposed that EA at doses of 12.5 ($P<0.05$), 25 ($P<0.01$), 50 ($P<0.001$) and 100 mg/kg ($P<0.001$) significantly increased the grooming activity time in comparison with vehicle-treated group. Our findings demonstrated that administration of EA at dose of 100 mg/kg significantly reduced the horizontal activity when compared with vehicle-received mice ($P<0.05$) (Fig 4).

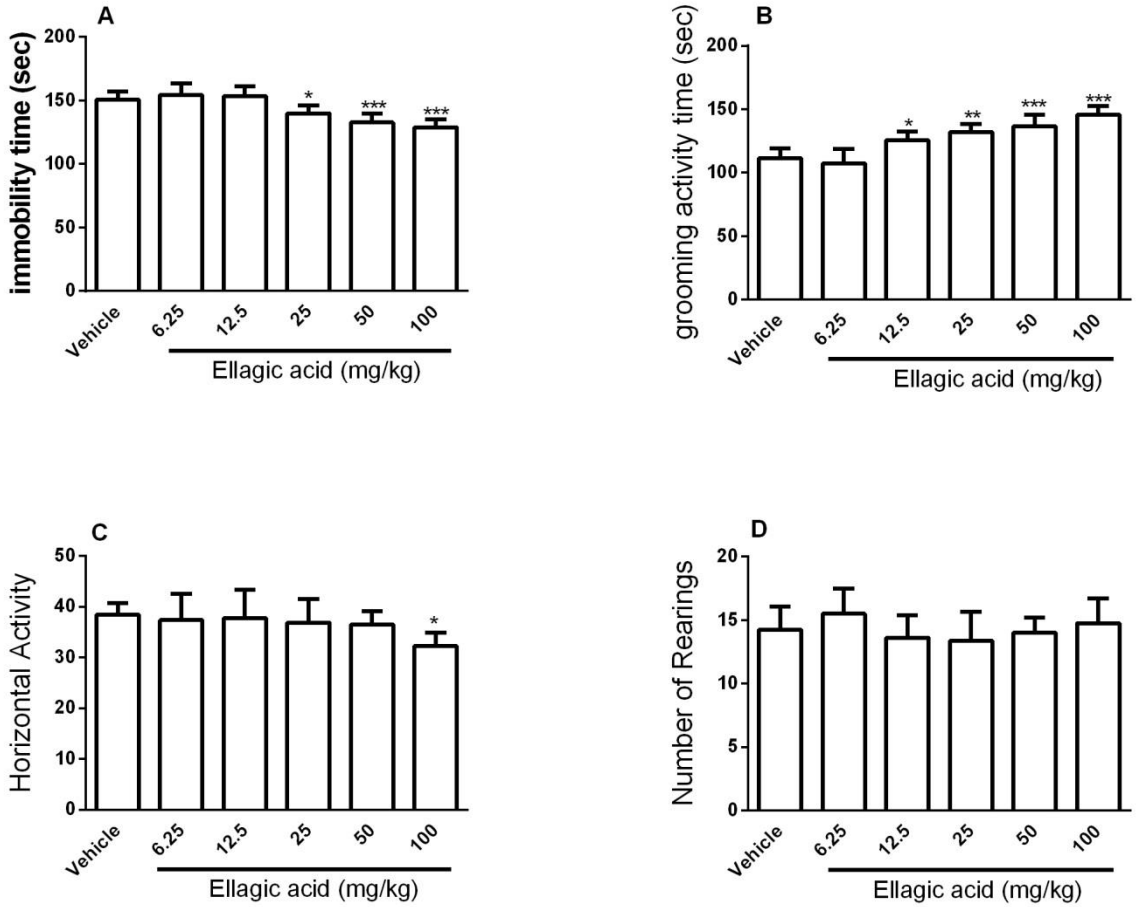


Fig. 4. Effect of administration of the EA at different doses on depressive -like behaviors in adult male mice, FST (A), splash test (B), horizontal activity in the OFT (C) and number of rearings in the OFT (D). Values are presented as the mean ± S.E.M from 8 animals and were analyzed one-way ANOVA. *P<0.05, **P<0.01 and ***P<0.001 compared with the vehicle-treated mice.

Effects of inhibitor and precursor of NOS on the depressive-like behaviors

One-way ANOVA revealed that there are significant difference among experimental groups in the FST ($F(4, 35) = 12.04$, $P<0.001$), splash test ($F(4, 35) = 41.11$, $P<0.001$), horizontal activity in the OFT ($F(4, 35) = 0.6861$, $P>0.05$). However there is no significant difference among groups in the vertical activity of the OFT ($F(4, 35) = 1.542$, $P>0.05$) (Fig 5). Post- test analysis revealed that administration of L-NAME at dose of 10 mg/kg

significantly reduced the immobility time in the FST (P<0.05) in compared with the vehicle-treated mice. In addition administration of the L-arg at dose of 50 mg/kg significantly increased the immobility time in the FST (P<0.05).

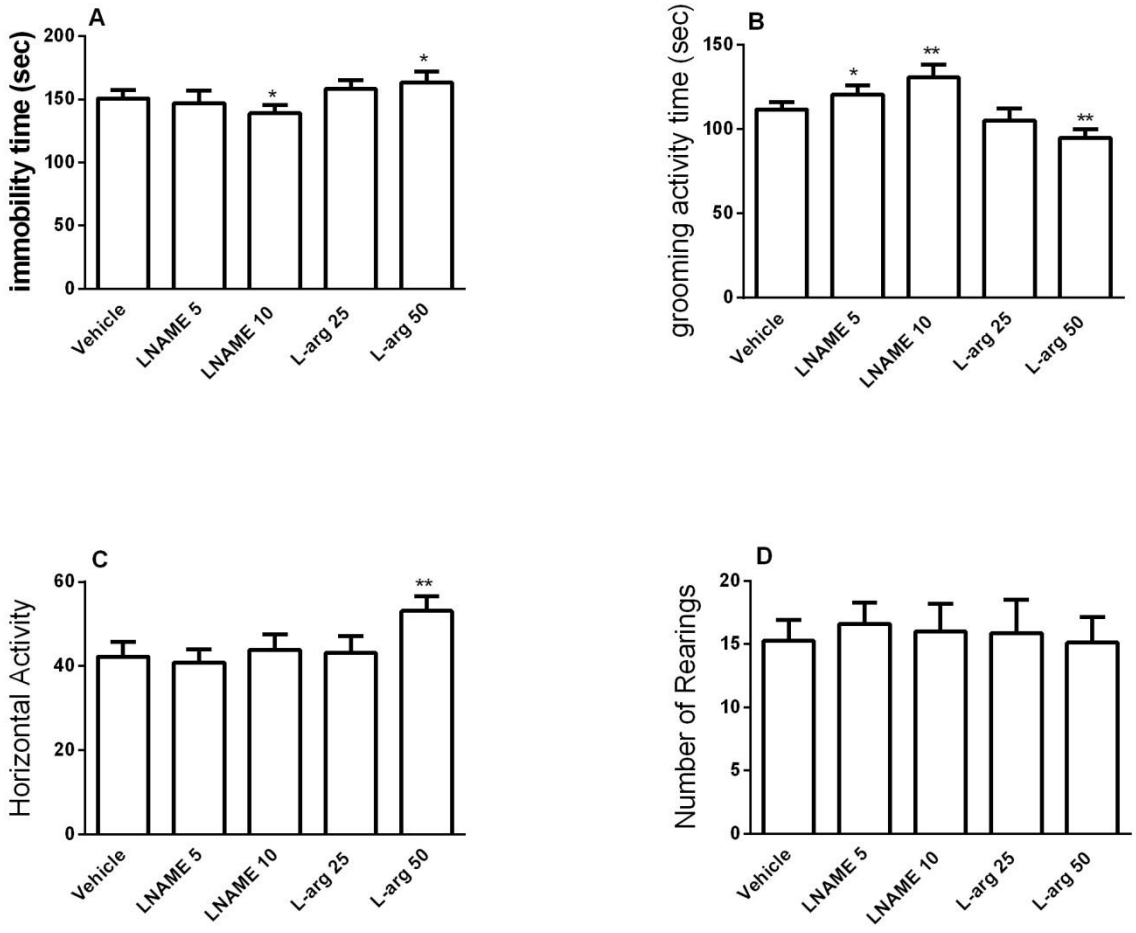


Fig. 5. Effect of administration of the NOS inhibitor (L-NAME) and NO precursor (L-arg) at different doses on depressive -like behaviors in adult male mice, FST (A), splash test (B), horizontal activity in the OFT (C) and number of rearings in the OFT (D). Values are presented as the mean ± S.E.M from 8 animals and were analyzed one-way ANOVA. *P<0.05 and **P<0.01 compared with the vehicle-treated mice.

Effects of agonist and antagonists of NMDA-R on the depressant-like behaviors

One-way ANOVA revealed that there are significant difference among experimental groups in the FST ($F(4, 35) = 74.10$, $P<0.001$), splash test ($F(4, 35) = 21.00$, $P<0.001$), vertical activity in the OFT ($F(4, 35) = 4.290$, $P<0.05$). However there is no significant difference among groups in the horizontal activity of the OFT ($F(4, 35) = 1.383$, $P>0.05$) (Fig 6). Results determined that NMDA agonist (150 mg/kg) significantly increased the immobility time in the FST in comparison with control counterpart ($P<0.01$). Furthermore, administration of ketamine at doses of 0.2 mg/kg ($P<0.01$) and 0.5 mg/kg ($P<0.001$) significantly decreased the immobility time in the FST in comparison with vehicle-received group. In case of splash test, we found that NMDA agonist (150 mg/kg) significantly reduced the grooming activity time ($P<0.01$) and ketamine at dose of 0.5 mg/kg significantly increased the grooming activity time in compared with vehicle -treated counterpart. In addition we observed that ketamine at dose of 0.5 mg/kg reduced the number of rearings (vertical activity) in compared with the vehicle group ($P<0.05$).

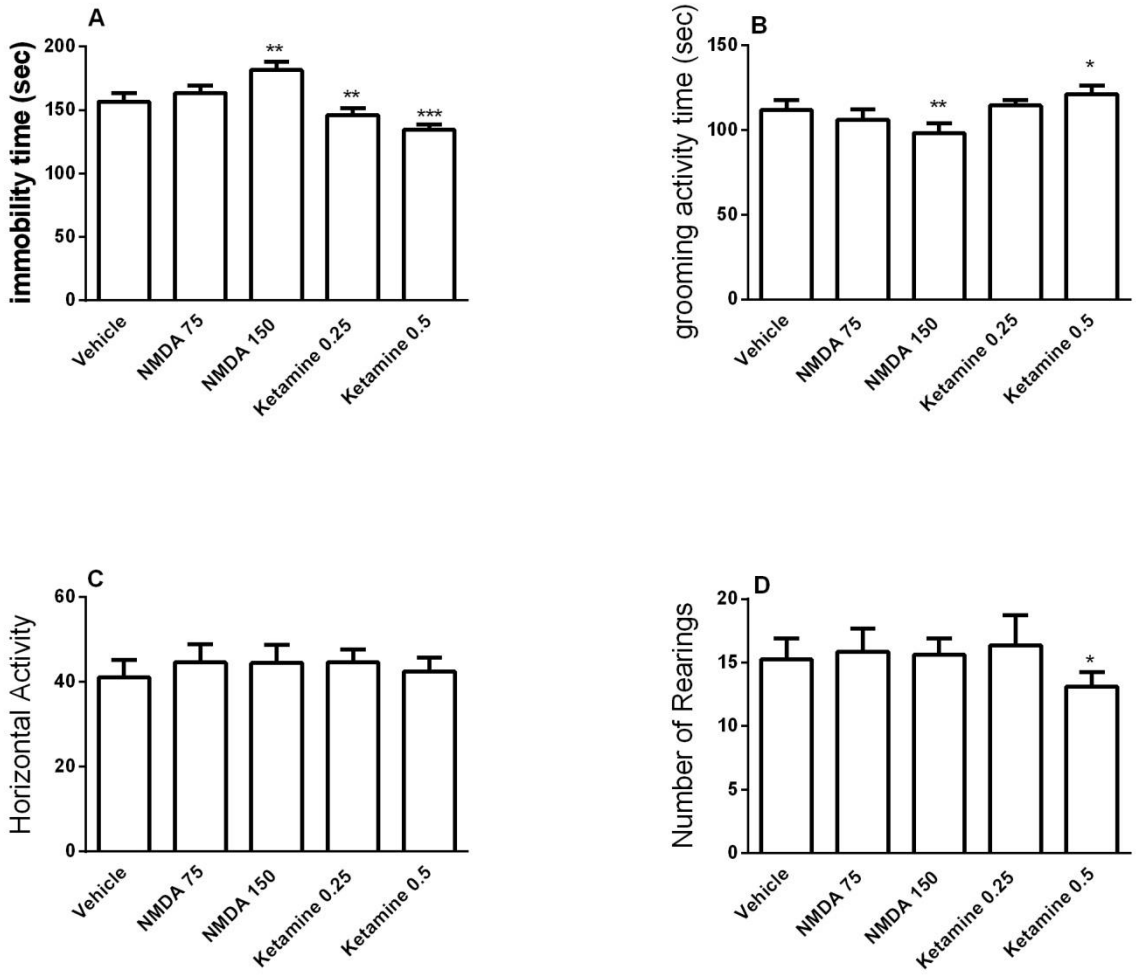


Fig. 6. Effect of administration of the NMDA agonist (NMDA) and NMDA antagonist (ketamine) at different doses on depressive -like behaviors in adult male mice, FST (A), splash test (B), horizontal activity in the OFT (C) and number of rearings in the OFT (D). Values are presented as the mean ± S.E.M from 8 animals and were analyzed one-way ANOVA. *P<0.05, *P<0.01 and ***P<0.001 compared with the vehicle -treated mice.

Involvement of NO in the antidepressant-like effects of EA

Two-way ANOVA revealed that there are significant difference among experimental groups in the FST ($F(6, 42) = 11.88$, $P<0.001$) and the splash test ($F(6, 42) = 33.37$, $P<0.001$) (Fig 7). However there is no significant difference

among groups in the horizontal activity of the OFT ($F(6, 42) = 3.49$, $P>0.05$) and vertical activity in the OFT ($F(6, 42) = 2.415$, $P>0.05$).

Post- test analysis determined that co-administration of sub-effective dose of L-NAME (5 mg/kg) with sub-effective dose of EA (6.25 mg/kg) significantly decreased the immobility time in the FST ($P<0.01$). In this regard, co-administration of effective dose of EA (25 mg/kg) with L-arg (50 mg/kg) significantly increased the immobility time in the FST ($P<0.05$) when compared with the vehicle -treated counterpart. Furthermore, co-applied of the L-NAME with sub-effective dose of EA decreased the immobility time in compared with the group received EA at dose of 6.25 mg/kg ($P<0.05$). Co-administration of the L-arg with EA at dose of 25 mg/kg significantly increased the immobility time in compared with the group received EA at dose of 25 mg/kg ($P<0.01$).

In the splash test we observed that co-treatment of sub-effective dose of L-NAME (5 mg/kg) with sub-effective dose of EA (6.25 mg/kg) significantly increased the grooming activity time ($P<0.01$). As, co-administration of effective dose of EA (25 mg/kg) with L-arg (50 mg/kg) significantly decreased the grooming activity ($P<0.001$) when compared with the vehicle -treated counterpart. Co-applied of the L-NAME with sub-effective dose of EA increased the grooming activity time in compared with the group received EA at dose of 6.25 mg/kg ($P<0.05$). Co-administration of the L-arg with EA at dose of 25 mg/kg significantly decreased the grooming activity time in compared with the group received EA at dose of 25 mg/kg ($P<0.001$).

In addition we did not find significant differences amongst groups either in horizontal or vertical activities in the OFT.

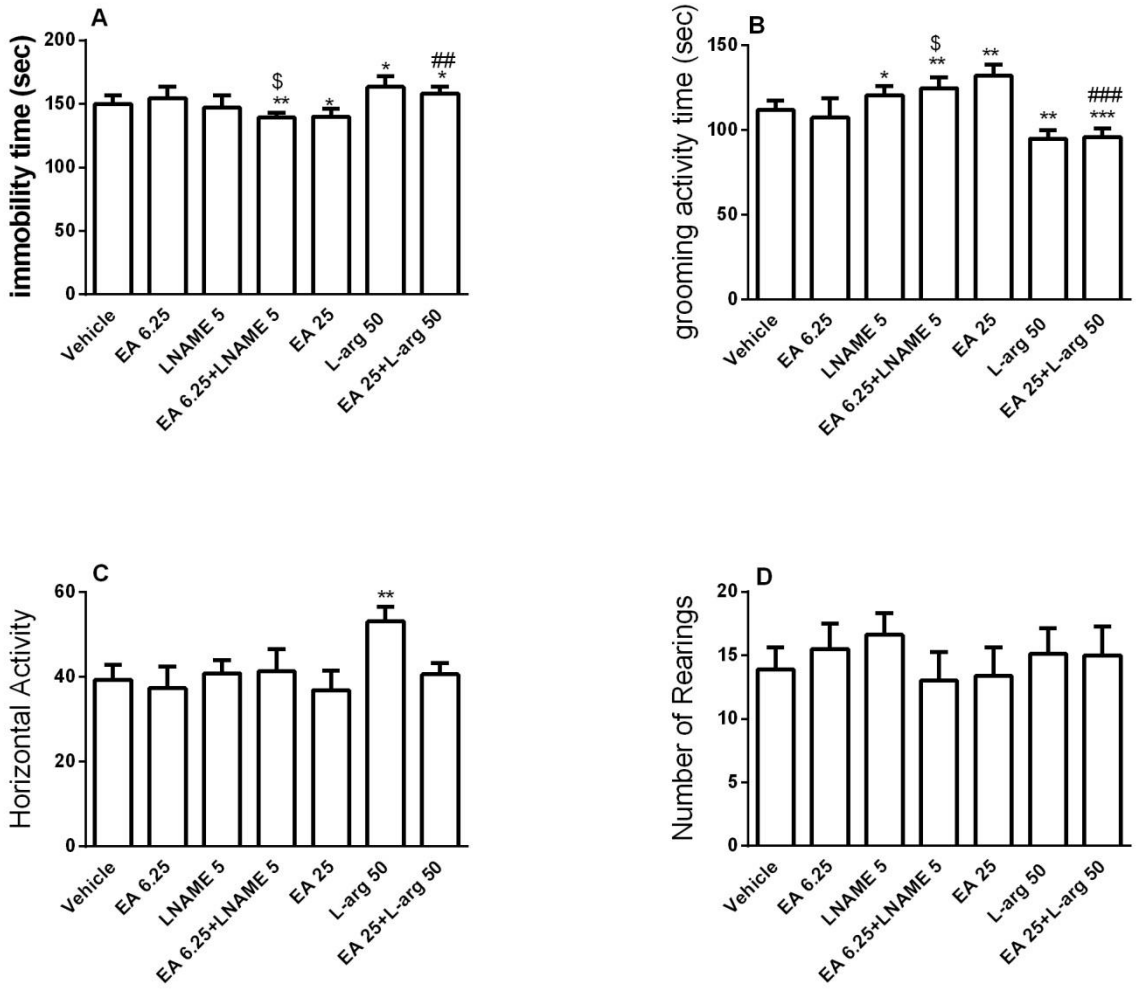


Fig. 7. Involvement of NO in antidepressant effects of EA on the immobility time in the FST (A), grooming activity in the splash test (B) and the horizontal activity (C) and vertical activity (D) in the OFT in adult male mice. Values are expressed as the mean ± S.E.M from 6 to 8 animals and were analyzed using two-way ANOVA followed by Tukey's post hoc test. *P<0.05, **P<0.01 and ***P<0.001 compared with the vehicle -treated counterpart, $P<0.05 compared with group received EA at dose of 6.25 mg/kg, ##P<0.01 and ###P<0.001 compared with mice treated with EA at dose of 25 mg/kg.

Involvement of NMDA in the antidepressant-like effects of EA

Two-way ANOVA revealed that there are significant difference among experimental groups in the FST (F (6, 42) = 40.63, P<0.001) and the splash test

(F (6, 42) = 35.61, P<0.001). However there is no significant difference among groups in the horizontal activity of the OFT (F (6, 42) = 2.533, P>0.05) and vertical activity in the OFT (F (6, 42) = 2.195, P>0.05) (Fig 8).

Post- test analysis determined that co-administration of sub-effective dose of ketamine (0.25 mg/kg) with sub-effective dose of EA (6.25 mg/kg) significantly decreased the immobility time in the FST (P<0.001). Furthermore, co-administration of effective dose of EA (25 mg/kg) with NMDA (150 mg/kg) significantly increased the immobility time in the FST (P<0.05) when compared with the vehicle -treated counterpart. Co-applied of the ketamine with sub-effective dose of EA decreased the immobility time in compared with the group received EA at dose of 6.25 mg/kg (P<0.05). Co-administration of the NMDA with EA at dose of 25 mg/kg significantly increased the immobility time in compared with the group received EA at dose of 25 mg/kg (P<0.01).

In the splash test we observed that co-treatment of sub-effective dose of ketamine (0.25 mg/kg) with sub-effective dose of EA (6.25 mg/kg) significantly increased the grooming activity time (P<0.001). In addition, co-administration of effective dose of EA (25 mg/kg) with NMDA (150 mg/kg) significantly decreased the grooming activity (P<0.001) when compared with the vehicle -treated counterpart. Co-applied of the ketamine with sub-effective dose of EA increased the grooming activity time in compared with the group received EA at dose of 6.25 mg/kg (P<0.01). Co-administration of the NMDA with EA at dose of 25 mg/kg significantly decreased the grooming activity time in compared with the group received EA at dose of 25 mg/kg (P<0.01).

In addition we did not find significant differences amongst groups either in horizontal or vertical activities in the OFT.

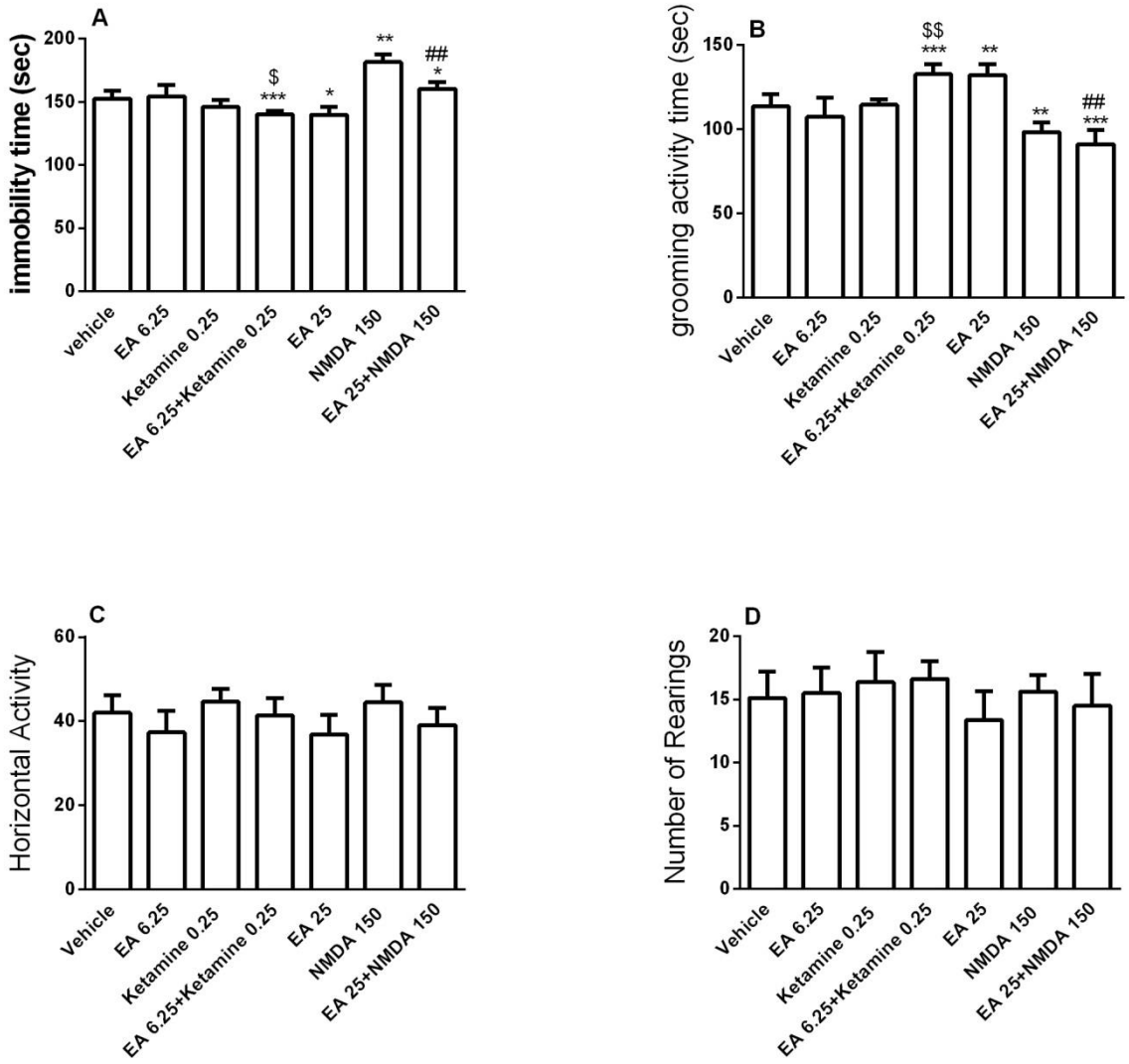


Fig. 8. Involvement of NMDA in anti-depressant- like effects of EA on the immobility time in the FST (A), grooming activity in the splash test (B) and the horizontal activity (C) and vertical activity (D) in the OFT in adult male mice. Values are expressed as the mean ± S.E.M from 8 animals and were analyzed using two-way ANOVA followed by Tukey's post hoc test. *P<0.05, and ***P<0.001 compared with the vehicle- treated counterpart, $P<0.05 and $$P<0.05 compared with group received EA at dose of 6.25 mg/kg, ##P<0.01 compared with mice treated with EA at dose of 25 mg/kg.

EA decreased the level of nitric oxide in the hippocampus

Two-way ANOVA exposed that there are significant difference among experimental groups in case of NO concentration in the hippocampus ($F(4, 20) = 17.42$, $P<0.001$) (Fig 9). Post- test analysis showed that administration of EA at dose of 25 mg/kg significantly reduced the level of NO in the hippocampus in compared with the vehicle -received group ($p<0.01$). Furthermore, we observed that co-administration of EA at effective dose of 25 mg/kg with L-arg (50 mg/kg) significantly increased the level of NO in the hippocampus when compared with the group received EA at dose of 25 mg/kg ($P<0.05$). We showed that co-administration of sub-effective dose of EA (6.25 mg/kg) with sub-effective dose of L-NAME (5 mg/kg) significantly decreased the NO level in the hippocampus in compared with the vehicle group ($P<0.01$) and the group received EA at dose of 6.25 mg/kg ($P<0.05$).

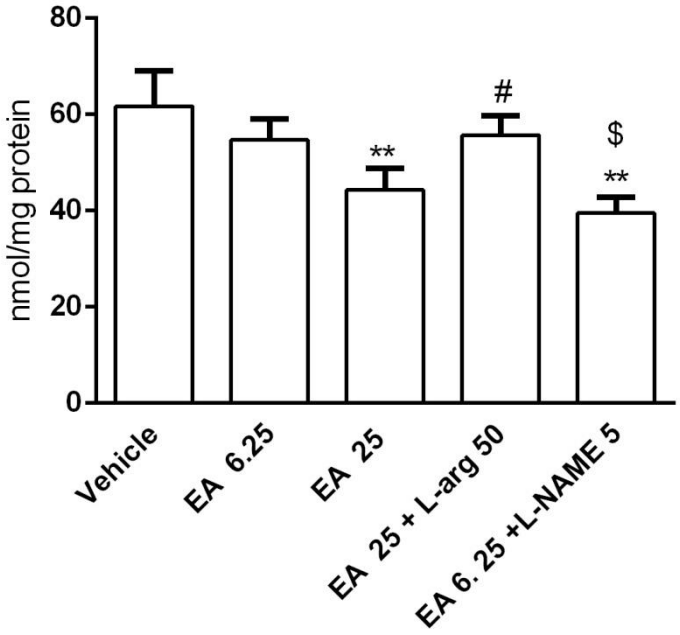
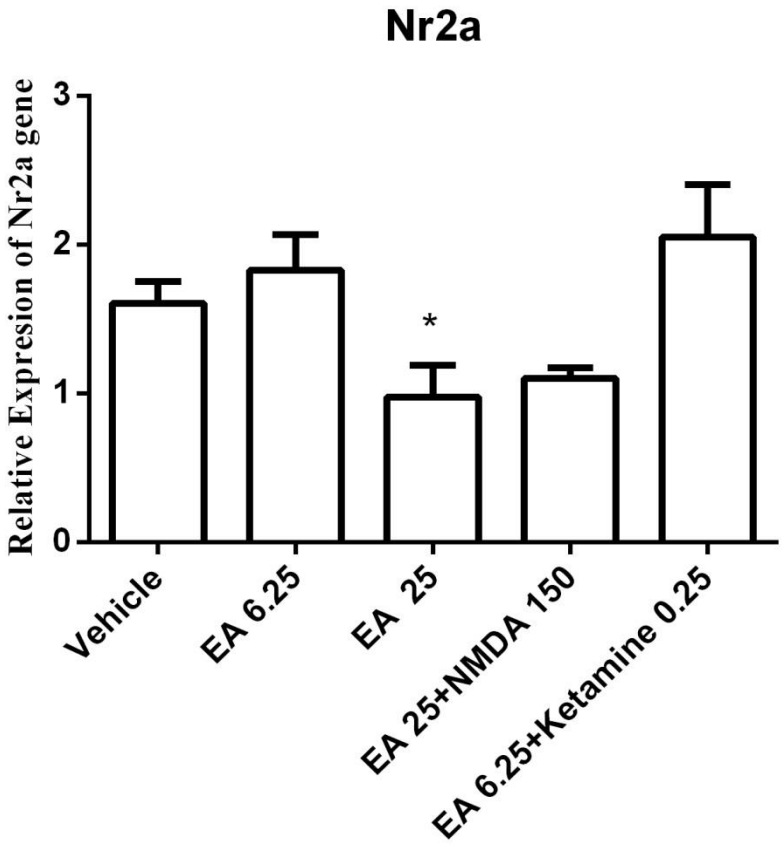


Fig. 9. Effect of EA and NOS inhibitor as well as NO precursor on the NO level in the hippocampus. Values are expressed as the mean ± S.E.M from 4 animals and were analyzed using two-way ANOVA followed by Tukey's post hoc test.

**P<0.05 compared with the vehicle -treated counterpart, # P<0.05 compared with the group received EA at dose of 25 mg/kg and $P<0.05 compared with the group received EA at dose of 6.25 mg/kg.

EA **modulates the expression of NR2A and NR2B subunits of the NMDA-R in the hippocampus**

Two- way ANOVA analysis showed that there are significant differences among experimental groups in case of expression of NR2A ($F(4, 12) = 5.143$, $P < 0.01$) and NR2B ($F(4, 12) = 4.696$, $P < 0.05$) in the hippocampus (Fig. 10). Post- test analysis showed that EA at dose of 25 mg/kg significantly decreased the expression of NR2A subunit of the NMDA-R in comparison with the control group (P<0.05). Also, we determined that administration of the EA at dose of 6.25 mg/kg significantly increased the expression of the NR2B receptor in compared with the control group (P<0.05). Furthermore, co-administration of the ketamine (sub-effective dose of 0.25) with sub-effective dose of EA (6.25 mg/kg) significantly decreased the expression of the NR2B receptor in compared with the group- received EA at dose of 6.25 mg/kg.



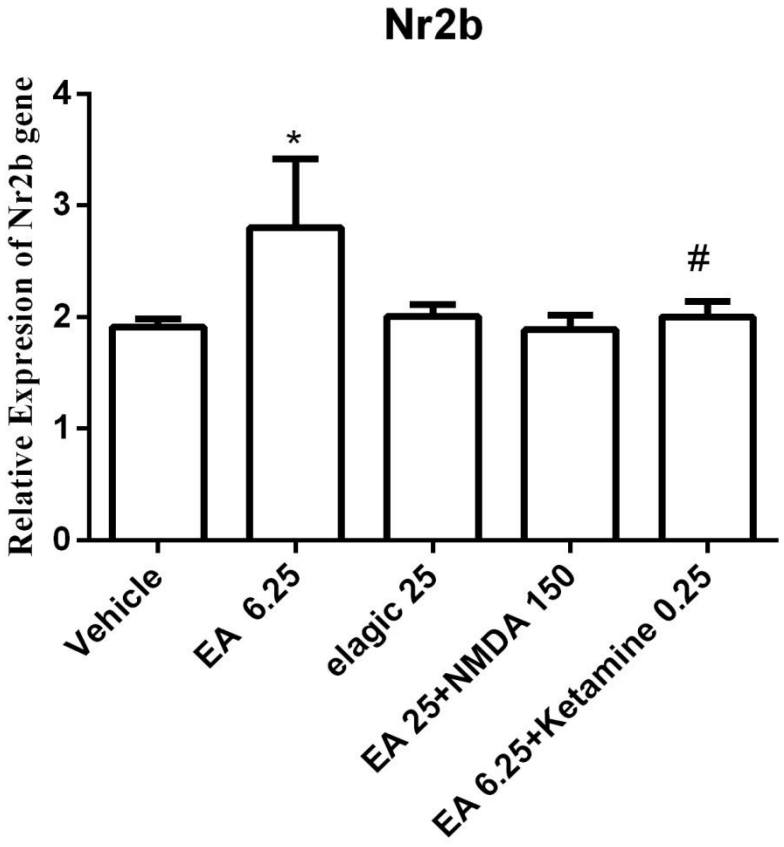


Fig 10. The expression of NR2A and NR2B subunits of the NMDA-R in the hippocampus. Values are expressed as the mean ± S.E.M from 4 samples and were analyzed using two-way ANOVA followed by Tukey's post hoc test. *P<0.05 compared with the normal group and $^{\#}$P<0.05 compared with the group received EA at dose of 6.25 mg/kg.

Discussion

Results of the present study showed that EA in a dose dependent manner exerted antidepressant-like effects in the valid behavioral tests related to depression including FST, splash test. We observed that administration of L-NAME similar to ketamine produced antidepressant-like effects while treatment with L-arg such as NMDA caused a depressive-like behavior in mice. In order to determine the possible mechanisms of action of the EA, EA in combination with L-NAME, L-arg, ketamine and NMDA was administered. Findings determined that at least in part, NMDA-NO pathways mediated the antidepressant-like effect of EA in mice.

FST is a valid screening test for the evaluation of new antidepressants. In the FST, the increase in immobility time of rodents indicating depressive-like behavior (Cryan and Holmes 2005, Haj-Mirzaian, Amini-Khoei et al. 2017). In this test increase in immobility time reflects the inability of mice to deal with an acute unescapable challenge expressing the depressive-like behaviors. The splash test is a recognized valid test assessing the self-care difficulties and motivation in rodents. In the splash test a poor response to sucrose 10% as decrease in the grooming time representing a disorder in motivation and self-care behaviors (Amini-Khoei, Mohammadi-Asl et al. 2017). The decrease in the grooming activity time reflects depressive-like behavior. In addition, we performed the OFT to confirm that locomotor activity of mice following

treatments does not affect the FST results, and the immobility of animals in the FST is not allied with their hypo-locomotion (Haj-Mirzaian, Amiri et al. 2018). In the other word, OFT was conducted immediately before the FST to assess ambulatory behavior of mice and approve that disparities which happen in motor activity did not affect the duration of immobility in the FST.

EA, a chromene-dione derivative (2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione; C14H6O8, is a common metabolite in some plants, several fruits (pomegranates, persimmons, raspberries, black raspberries, wild strawberries, peaches, plums) and seeds (walnuts, almonds), and vegetables)Ríos, Giner et al. 2018(. Previous studies have been suggested several pharmacological effects for EA including anti-inflammatory, neuroprotective, and antioxidant properties (Seeram, Adams et al. 2005, de Oliveira 2016). It has been proposed that EA can scavenge damaging radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Priyadarsini, Khopde et al. 2002). *In vitro* studies reported that EA can dose dependently decrease the activation of inducible NOS (iNOS) in the stimulated macrophages and reduce the NO concentration in these cells (Kassim, Achoui et al. 2010). EA possessed neuro-protective effects in the brain against various damages. These defensive properties are resulted from positive effects on neuro-inflammatory responses and (pro) oxidant mediators such as NO, cytokines and superoxide radicals (de Oliveira 2016).

Few studies have been conducted on the antidepressant-like effects of EA in animal models. In this context studies have been demonstrated that monoaminergic system, nitric oxide pathway and brain derived neurotrophic factor (BDNF) are involved in the antidepressant-like effect of EA in rodents (Dhingra and Chhillar 2012, Girish, Raj et al. 2012, Bedel, Kencebay Manas et al. 2017). However, the precise mechanisms underlying the antidepressant-like effect of EA are not yet fully specified. Findings of the current study

demonstrated that EA dose dependently exerts antidepressant-like effects in the FST and the splash test. We showed that EA at doses of 12.5, 25, 50 and 100 mg/kg significantly decreases the immobility time in the FST as well as increased the grooming activity time in the splash test. However, EA at dose of 6.25 mg/kg possesses any positive effects in the related behavioral tests and this dose considered as sub-effective dose. These results are in agreement with previous studies which have been reported that EA possessed antidepressant-like effects in rodents (Dhingra and Chhillar 2012, Girish, Raj et al. 2012). In addition we found that EA, except at dose of 100 mg/kg has no effect on locomotor activity in the OFT.

Ample evidence demonstrated that glutamatergic system has critical role in the pathophysiology of psychiatrics such as depression. In this regards, both clinical and preclinical studies have showed that NMDA receptor antagonists exerted antidepressant (-like) effects (Berman, Cappiello et al. 2000, Tokita, Yamaji et al. 2012, Zorumski and Izumi 2012). Previously, it has been well-known that acute administration of ketamine possesses antidepressant-like effects in behavioral tests relevant to depression including FST and splash test (Maeng, Zarate Jr et al. 2008, Brachman, McGowan et al. 2016). Clinical investigations have been reported that treatment with ketamine at sub-anesthetic dose lead to rapid, strong, and relatively constant antidepressant effects in patients with treatment-resistant depression(Maeng and Zarate 2007, Denk, Rewerts et al. 2011, Rybakowski, Permoda-Osip et al. 2013). Furthermore, experimental researches have been determined that NMDA agonists provoked depressive-like behaviors in relevant behavioral tests in rodents (Ostadhadi, Ahangari et al. 2016, Ostadhadi, Norouzi-Javidan et al. 2017). In this study using valid behavioral tasks related to depression in rodents, we showed that administration of ketamine decreases immobility time in the FST and increases grooming activity time in the splash test. Moreover, treatment of mice with the NMDA

increased immobility time in the FST and decreased grooming activity time in the splash test. Our results are in line with abovementioned studies which have been clarified that NMDA receptor activation leads to depressive-like behavior in rodents. Our findings present that ketamine blocks NMDA receptors that may account for its antidepressant-like effects in male mice. In completing this hypothesis, our results showed that NMDA (150 mg/kg) can reverse the antidepressant-like effect of effective dose of the EA (25 mg/kg) as well as ketamine (0.25 mg/kg) can potentiated the antidepressant-like effect of sub-effective dose of the EA (6.25 mg/kg). Our results proposing that blockade of NMDA receptors potently, at least in part, contributes to antidepressant-like effects of the EA in male mice. Real time RT-PCR findings showed that administration of EA at dose of 25 mg/kg significantly decreases the of NR2A subunit of the NMDA-R. Administration of the EA at dose of 6.25 mg/kg significantly increased the expression of the NR2B receptor. Co-administration of the sub-effective dose of ketamine with sub-effective dose of EA (6.25 mg/kg) significantly decreased the expression of the NR2B receptor in compared with the group- received EA at dose of 6.25 mg/kg. Previous studies showed that alterations in NR2A and NR2B subunits of the NMDA-R involved in the pathophysiology of depression (Feyissa, Chandran et al. 2009, Zhang, Xu et al. 2013). We showed that co-applied of the ketamine with sub-effective dose of EA potentiated the antidepressant-like effect of sub-effective dose of the EA as co-administration of the NMDA with effective dose of the EA mitigated the antidepressant-like effect of effective dose of the EA. Given that, our results provided evidence that NMDA receptor complex can be considered as a target for discovery of novel antidepressants.

Nitric oxide as a neuromodulator has pivotal role in regulation of physiological functions in the CNS (Chong, Ai et al. 2018). Clinical studies have been determined that level of the NO significantly increases in the plasma

samples of MDD patients (Schiavone, Neri et al. 2016, Lu, Zhang et al. 2018). Previous studies have been demonstrated that NO can be considered as a biomarker for detection of depression. In this regards it has been shown that following treatment with antidepressant drugs the level of NO significantly reduced in response to treatment (Bersani, Wolkowitz et al. 2016, Loeb, Feve et al. 2017). Preclinical studies clarified that administration of NOS inhibitors led to antidepressant-like properties in behavioral tests related to depression in animals. In this regards literature said that inhibition of the NOS involves in the antidepressant-like effects of new agents with antidepressant-like properties (Ostadhadi, Ahangari et al. 2016, Ostadhadi, Khan et al. 2016). Findings of the current study showed that administration of the L-NAME exerts antidepressant-like effects in the FST and the splash test manifested by decreasing in the immobility time and increasing in the grooming activity time, respectively. Furthermore, we observed that administration of the L-arg led to depressive-like behaviors in the FST and splash test. In addition we observed that EA significantly decreases the NO level in the hippocampus and in this concept L-arg reversed and L-NAME potentiated the effect of sub-effective dose of EA on the hippocampal NO level. These results are in line with aforementioned previous studies which conformed that NOS inhibitors causes antidepressant-like behavior and NO precursors provoked depressive-like actions. To support this, we evaluated the effect of combination therapy between EA and NO mediators. We showed that co-treatment of sub-effective dose of EA (6.25 mg/kg) with sub-effective dose of L-NAME (5mg/kg) significantly leads to antidepressant-like behaviors in the FST and splash test. In addition, we observed that co-administration of effective dose of the EA (25 mg/kg) with l-arg (50 mg/kg) significantly reverses the positive effect of the EA as shown by increasing in immobility time in the FST and decreasing in grooming activity time in the splash test. In our study, co-applied L-NAME with sub-effective dose of EA potentiated the antidepressant-like effect of the sub-effective dose of

the EA as co-administration of L-arg with effective dose of the EA significantly alleviated the antidepressant-like effect of the EA. Given that**,** our results suggest that attenuation of NO level potently, at least in part, contributes to antidepressant-like effects of the EA in male mice. Given that, our findings demonstrated that NMDA-NO pathway, partially at least involves in the antidepressant-like effect of EA in behavioral tests related to depression including FST and splash test.

Conclusion

In conclusion, findings of the present study provides evidence that, 1) EA dose dependently possesses antidepressant-like effects in behavioral tests related to depression, 2) NO is involved in the antidepressant-like effect of EA that way NOS inhibition potentiated the effect of sub-effective dose of EA and NO precursor mitigated the antidepressant-like effect of EA, 3) NMDA is involved in the antidepressant-like effect of EA that way ketamine potentiated the effect of sub-effective dose of EA and NMDA mitigated the antidepressant-like effect of EA.

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Conflicts of interest

The authors have no conflicts of interest to declare regarding the study described in this article and preparation of the article.

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Highlights

Ellagic acid (EA) exerted antidepressant-like effect in mice

Nitric oxide synthase inhibitor (L-NAME) possessed antidepressant-like effect

NMDA receptor antagonist (ketamine) exerted antidepressant-like effect

Sub-effective doses of the L-NAME and ketamine potentiated the antidepressant-like effect of sub-effective dose of EA

Nitric oxide synthase inhibitor (L-arg) as well as NMDA receptor agonist reversed antidepressant-like effect of effective dose of EA

EA decreased NO level as well as NR2A expression in the hippocampus