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Tropisetron attenuated the anxiogenic effects of social isolation by modulating nitrergic system and mitochondrial function☆

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ABSTRACT

Background: Early social isolation stress (SIS) is associated with the occurrence of anxiety behaviors. It seems interaction between the nitrergic system and mitochondrial function plays a role in mediating the anxiety-like behaviors. In this study, we aimed to investigate the anxiolytic effects of tropisetron in animal model of SIS and we try to illustrate the possible role of nitrergic system and mitochondrial function.
Methods: We applied early social isolation paradigm to male NMRI mice. Animals treated with various doses of tropisetron, nitric oxide agents or their combination and anxiety-like behaviors of animals were assessed using valid behavioral tests including elevated plus maze (EPM), open-field test (OFT) and hole-board test (HBT) in their adulthood. Effects of housing conditions and drug treatments on the mitochondrial function were investigated in the hippocampus by assessing the ATP, GSH, ROS and nitrite levels.
Results: Anxiogenic effects of early SIS were assessed in the EPM, OFT, and HBT. Also, SIS disrupted mitochondrial function and caused oxidative stress in the hippocampus of stressed animals. Tropisetron showed an anxiolytic effect in the stressed mice. Also, these effects were mediated by nitrergic system by affecting mitochondrial function and modulating the oxidative stress. L-arginine, a nitric oxide precursor, abolished the anxiolytic effects of tropisetron in the behavioral tasks and blocked the protective effects of it against mitochondrial and oxidative challenge.
Conclusions and general significance: Our results demonstrated tropisetron attenuated the anxiogenic effects of SIS by mitigation of the negative effects of nitric oxide on mitochondrial function.
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1. Introduction

Anxiety and stress disorders are common mental illnesses with high prevalence and comorbidity [1,2]. Experiencing aversive events in early stages of life negatively affects the behavior and brain development and also, is regarded as a putative risk factor for vulnerability to psychiatric disorders such as affective disorders [3,4]. A substantial body of evidence indicates that applying social isolation stress (SIS) to rodents induces a variety of long-lasting behavioral disturbances relevant to stress such as anxiety-like behaviors [5,6]. In this regard, underlying mechanisms through which SIS induces anxiety-like behaviors are not clearly understood. Increasing lines of evidence indicate that mitochondrial dysfunction [7], oxidative and nitrosative stress (O&NS) also contribute to pathogenesis of anxiety-like disorders [8–10]. Evidence indicates that SIS-induced O&NS contributes to behavioral and neurochemical alterations in rodents [11,12]. Under stressful conditions, mitochondria generate excessive amounts of reactive oxygen species (ROS), which correlates with glutathione (GSH) and ATP depletion, and consequently oxidative damage [13–15]. Additionally, it has been reported that anxiolytic drugs decrease the O&NS in stressed animals [9]. Moreover, overproduction of nitric oxide (NO) in the stressful conditions has been reported to induce anxiety-like behaviors [16] that administration of aminoguanidine (specific inhibitor of inducible

Abbreviations: SIS, social isolation stress; O&NS, oxidative and nitrosative stress; AG, aminoguanidine; HBT, hole-board test; OFT, open-field test; EPM, elevated plus maze

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nitric oxide synthase or iNOS) or L-NAME (non-specific inhibitor of NOS) reversed the anxiety-like responses [17,18].Also, NO-induced O&NS causes mitochondrial dysfunction and cell injury [19].

On the other hand, a number of studies have reported that impairment in neurotransmitter systems (mostly serotonergic system) plays a role in development of aggression, anxiety and fear in socially isolated rodents [20–22]. Evidence suggests that 5-hydroxytryptamine3 (5-HT3) receptors, as ligand gated ion channels, are involved in development and maturation of the brain mostly formation of the inhibitory networks. Also, 5-HT3 receptors have been reported to contribute to pathophysioliogy of anxiety and mood disorders that mice lacking these receptors exhibit reduced anxiety-like behaviors [23]. In this regard, several lines of research have demonstrated that tropisetron, a 5-HT3 antagonist, exhibits anxiolytic effects in both clinical and preclinical studies [24,25]. According to our recent studies, we found that tropisetron possesses protective properties against O&NS in pathologic conditions. In this context, we showed that tropisetron is able to attenuate O&NS as well as inflammatory responses in animal models of Alzheimer's disease and stroke [26,27]. In addition, recent studies have reported the antidepressant-like properties of 5-HT3 antagonists (including tropisetron) in both non-stressed and stressed animals [28–30]. Considering that mitochondrial performance and O&NS were reported as underlying mechanisms involved in pathogenesis of anxiety disorders, we tested the hypothesis that whether tropisetron is able to decrease anxiogenic effects of early SIS via regulating the mitochondrial performance. In this study, we applied early SIS paradigm because it has been suggested as a reliable and valid animal model to investigate the negative impacts of social environment (such as chronic stress) on neurobehavioral and neurochemical changes which similarly were observed in psychiatric disorders in humans [31,32].

2. Materials and methods

2.1. Animals

Male NMRI mice (Pasteur Institute, Tehran, Iran), weighing 10–12 g and in the postnatal day (PND: 21–23) were housed for 4 weeks under two different conditions: 1) social condition (SC) and 2) isolated condition (IC). Socially conditioned animals were housed in groups (6 mice per cage: 25 × 25 × 15 cm) while IC mice were housed individually in Plexiglas boxes (24 × 17 × 12 cm) under standard laboratory conditions (free access to food and water, temperature: 22 ± 2 °C, and 12-h light–dark cycle). All procedures in this work were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication #80-23) and institutional guidelines for animal care and use (Department of Pharmacology, School of Medicine, TUMS). Each experimental animal group consists of 6–9 in behavioral assessments, and 3–6 in molecular evaluations.

2.2. Drugs

The following drugs were used in this study: L-arginine (L-arg, a NO precursor), NG-nitro-L-arginine methyl ester (L-NAME, a non-selective NOS inhibitor), aminoguanidine (AG, a selective iNOS inhibitor) (Sigma, UK) and tropisetron (a selective 5-HT3 antagonist) (Sigma, St. Louis, MO, USA). All drugs were dissolved in saline and were administered intraperitoneally (i.p.) in the volume of 5-ml/kg animal weights. Doses of drugs were chosen according to the previous studies as well as our pilot studies (described below), we treated mice with L-arg (30 min), AG and L-NAME (45 min) and tropisetron (60 min) prior to behavioral or molecular experiments [17,33].

2.3. Experimental design

After 4 weeks of housing under isolation or social conditions, animals (PND: 52) were subjected to behavioral tests. In the first part of the experiment, effects of housing conditions on the anxiety-like behaviors were investigated using behavioral tests which are considered as valid methods for assessing anxiety in rodents including open field test (OFT), hole board test (HBT), and elevated plus maze test (EPM) [34–36].

In the next step, we investigated the possible effect of tropisetron as well as NO agents in mediating the anxiety-like behaviors of animals. In this regard, different sets of SC and IC mice were treated with different doses of following drugs: tropisetron (1, 3, and 5 mg/kg), L-NAME (5, 10 mg/kg), AG (20, 50 mg/kg), and L-arg (50, 100 mg/kg). Doses of each drug were chosen according to the pilot treatments, which were published in previous studies [31,33,37]. After administration of drugs, SC animals and IC animals were subjected to mentioned behavioral tasks. To exclude the possible effect of saline administration on animal behavior, SC and IC groups were injected with 5-ml/kg physiological saline. In order to investigate the role of nitrergic system in mediating the anxiolytic effect of tropisetron, subeffective doses of L-NAME and AG co-administered with subeffective dose of tropisetron to both SC and IC mice. Also, we co-administered subeffective dose of L-arg with effective dose of tropisetron. In the next step, we investigated the effects of different housing conditions and drug treatments on the hippocampal levels of glutathione (GSH), nitrite, and ATP and ROS production in the different sets of animals.

2.4. Behavioral tests

2.4.1. Open-field test (OFT)

The OFT was used to evaluate the anxiety-like behaviors of mice in response to various treatments and housing conditions [38]. The OFT apparatus was made of white opaque Plexiglas (50 cm × 50 cm × 30 cm) which was dimly illuminated. Mice were placed individually on the central zone of OFT box (30 cm × 30 cm) and spent time in the central area was recorded by a camera for a 5 min period. The apparatus was cleaned with 70% ethanol after testing each mouse.

2.4.2. Elevated plus maze (EPM)

The elevated plus maze (EPM) is an appropriate test to assess the effects of both anxiogenic and anxiolytic agents in rodents [36]. The apparatus was made of black opaque Plexiglas and consisted of two open (30 × 5 cm) and closed (30 × 5 × 15 cm) arms, which were connected by a platform area (5 × 5 cm). Testing room was dimly illuminated and animals were individually placed in the center of the EPM facing to closed arm and each behavioral session was videotaped for a 5 min period. The apparatus was cleaned with 70% ethanol after testing each mouse. The total time spent in the open arms, and number of entries into the open arms were recorded over a period of 5 min and reported as percentages.

2.4.3. Hole-board test (HBT)

Hole-board test is a reliable test to determine the anxiogenic/anxiolytic state in mice [38]. The apparatus was made of a white Plexiglas square (50 cm × 50 cm) with 16 equally sized holes (3 cm in diameter) and was positioned 50 cm above the floor. Mice were placed in the center of the board, and the number of head-dips was counted in a 5 min period. Decrease in number of head-dips was considered as anxiety-like behavior in animals. The apparatus was cleaned with 70% ethanol after testing each mouse.

2.5. Molecular assessments

2.5.1. Glutathione (GSH) measurement

Animals were decapitated under mild anesthesia, and then the hippocampi were dissected on ice-cold surface and immediately immersed in liquid nitrogen. Samples were centrifuged at 3000 g for 10 min at 4 °C, and the supernatant were collected. Glutathione levels were determined using 5, 50-dithiobis-(2-nitrobenzoic acid) or DTNB as the

indicator and spectrophotometer method. 0.1 mL of supernatant was added into 0.1 mol L^{-1} of phosphate buffer and 0.04% DTNB in a total volume of 3.0 mL (pH 8.9). The developed color was measured at 412 nm using a spectrophotometer (UV-1601 PC, Shimadzu, Japan). GSH content was expressed as μg mg^{-1} protein [39].

2.5.2. ATP assay

ATP levels were measured using luciferase enzyme as described in our previous work (39). Bioluminescence intensity was measured using Sirius tube luminometer (Berthold Detection System, Germany).

2.5.3. Measurement of reactive oxygen spices (ROS) formation

2,7-Dichlorodihydrofluorescein diacetate was used to measure ROS production in samples as reported by Vejražka et al. [40]. The sample was incubated with 5 μM DCF-DA at 30 °C for 15 min in the dark. Then, fluorescence was read with 485 nm excitation and 520 nm emission using a fluorimeter. Data were presented per milligram protein of tissue homogenate.

2.5.4. Nitrite assay

Nitrite measurement was carried out using the method, which was described in our previous studies. In brief, we measured nitrite levels as the result of the NO end product and, nitrite levels were determined using a colorimetric assay based on the Griess reaction. Concentration of nitrite was determined by reference to a standard curve of sodium nitrite (Sigma, USA) and normalized to the weight of each sample [41,42].

2.6. Statistics

Comparison between groups was analyzed using *t*-test, one-way and two-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant. The factors were housing [social condition (SC) and isolation condition (IC)] and treatments [control: no treatment and treatment: drug-administered animals by i.p. injection] for all assessments.

3. Results

3.1. Effects of housing conditions on anxiety-like behaviors

t-Test analysis revealed that applying SIS to mice in post weaning state induced anxiety-like behaviors in IC mice when compared to SC animals. In the OFT, SIS significantly decreased the spent time in the central zone in IC mice when compared to SC mice ($P < 0.01$, Fig. 1A). In the HBT, IC mice showed a decrease in number of head-dips in comparison with SC animals ($P < 0.001$, Fig. 1B). In the EPM, percentage of spent time in the open arms and percentage of open arms entries were evaluated as variables relevant to anxiety-like behaviors. In comparison with SC mice, SIS remarkably decreased percentage of spent time in the open arms ($P < 0.01$, Fig. 1C) as well as percentage of open arms entries ($P < 0.001$, Fig. 1D) in IC mice.

3.2. Effects of tropisetron on the anxiety-like behaviors in animals

In order to determine the subeffective doses of tropisetron in the animals, we assessed the effects of various doses of tropisetron (1, 3 and 5 mg/kg, i.p.) on anxiety-like behaviors in both SC and IC mice using the aforementioned tasks. One-way ANOVA demonstrated that treatment with all doses of tropisetron produced no significant alterations in anxiety-like behaviors in SC mice when compared with saline-treated SC mice in the OFT ($F_{(4, 25)} = 0.2119$, $P > 0.05$, Fig. 2A), HBT ($F_{(4, 35)} = 0.2465$, $P > 0.05$, Fig. 2C), and in the EPM ($F_{(4, 35)} =$

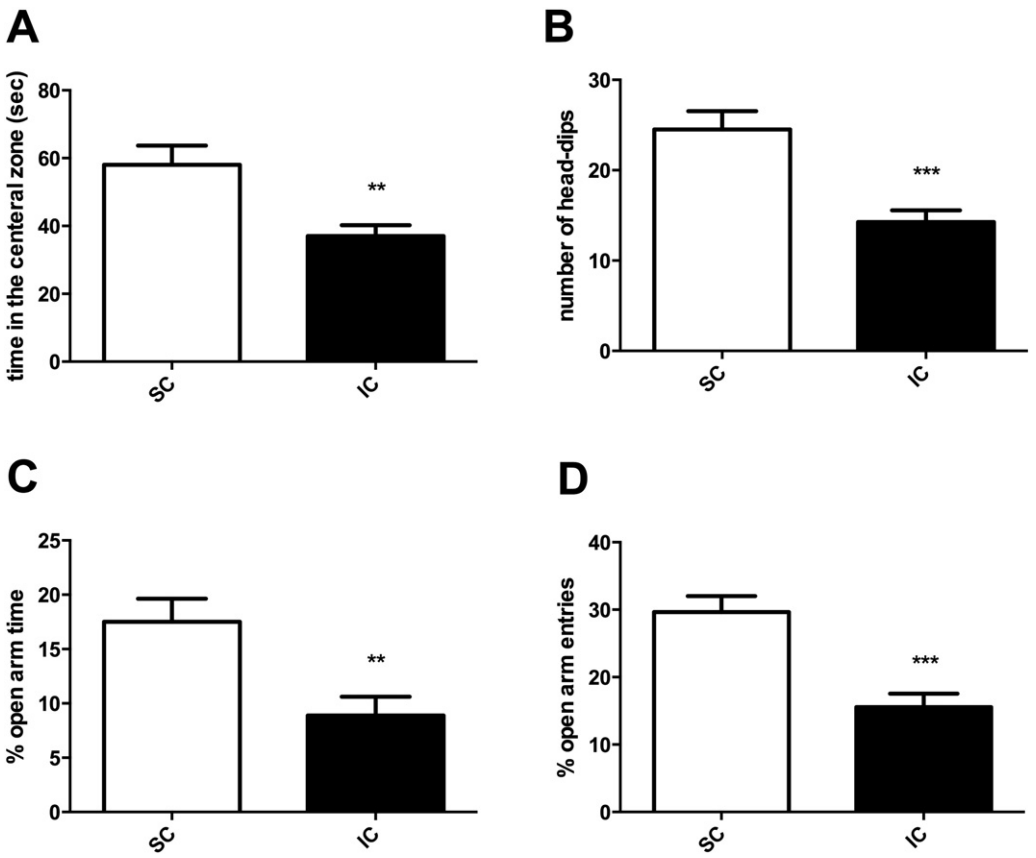

Fig. 1. Effect of different housing conditions, social condition (SC) and isolated condition (IC), on the time spent in the central zone in the OFT (A), number of head-dips in the HBT (B), the percentage of time spent in the open arm in the EPM (C), and the percentage of entries in the open arm in the EPM (D) in male mice. Values are expressed as the mean ± S.E.M from 6 to 8 animals and were analyzed using t-test. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the SC mice.

0.0658, $P > 0.05$, Fig. 2E) ($F_{(4, 40)} = 0.2451$, $P > 0.05$, Fig. 2G). However, ANOVA analysis revealed that same treatments changed the anxiety-like behaviors in IC mice in the OFT ($F_{(4, 25)} = 8.439$, $P < 0.001$, Fig. 2B), HBT ($F_{(4, 35)} = 6.853$, $P < 0.001$, Fig. 2D), and EPM ($F_{(4, 35)} = 4.378$, $P < 0.01$, Fig. 2F) ($F_{(4, 40)} = 6.251$, $P < 0.001$, Fig. 2H). In the OFT, Tukey's analysis showed that administration of tropisetron (3 and 5 mg/kg, but not 1 mg/kg) to IC mice significantly increased spent time in the central zone in comparison with saline-treated IC mice ($P < 0.05$ and $P < 0.001$). In the HBT, Tukey's analysis showed that administration of tropisetron (3 and 5 mg/kg, but not 1 mg/kg) to IC mice significantly increased the number of head-dips when compared to saline-treated IC mice ($P < 0.05$ and $P < 0.001$). In the EPM, only treatment with tropisetron 5 mg/kg increased the percentage of spent time in the open arms when compared to saline-treated IC mice ($P < 0.01$). In comparison with saline-treated IC mice, treatment with tropisetron (3 and 5 mg/kg, but not 1 mg/kg) increased the percentage of open arms entries in IC mice using Tukey's analysis ($P < 0.05$ and $P < 0.01$).

3.3. Effects of NO inhibitors/precursor on the anxiety-like behaviors in animals

In order to determine the effective and subeffective doses of NOS inhibitors and NO precursor in the animals, we investigated the effects of different doses of L-NAME, AG, and L-arg on the anxiety-like behaviors in both SC and IC mice using the aforementioned tasks. One-way

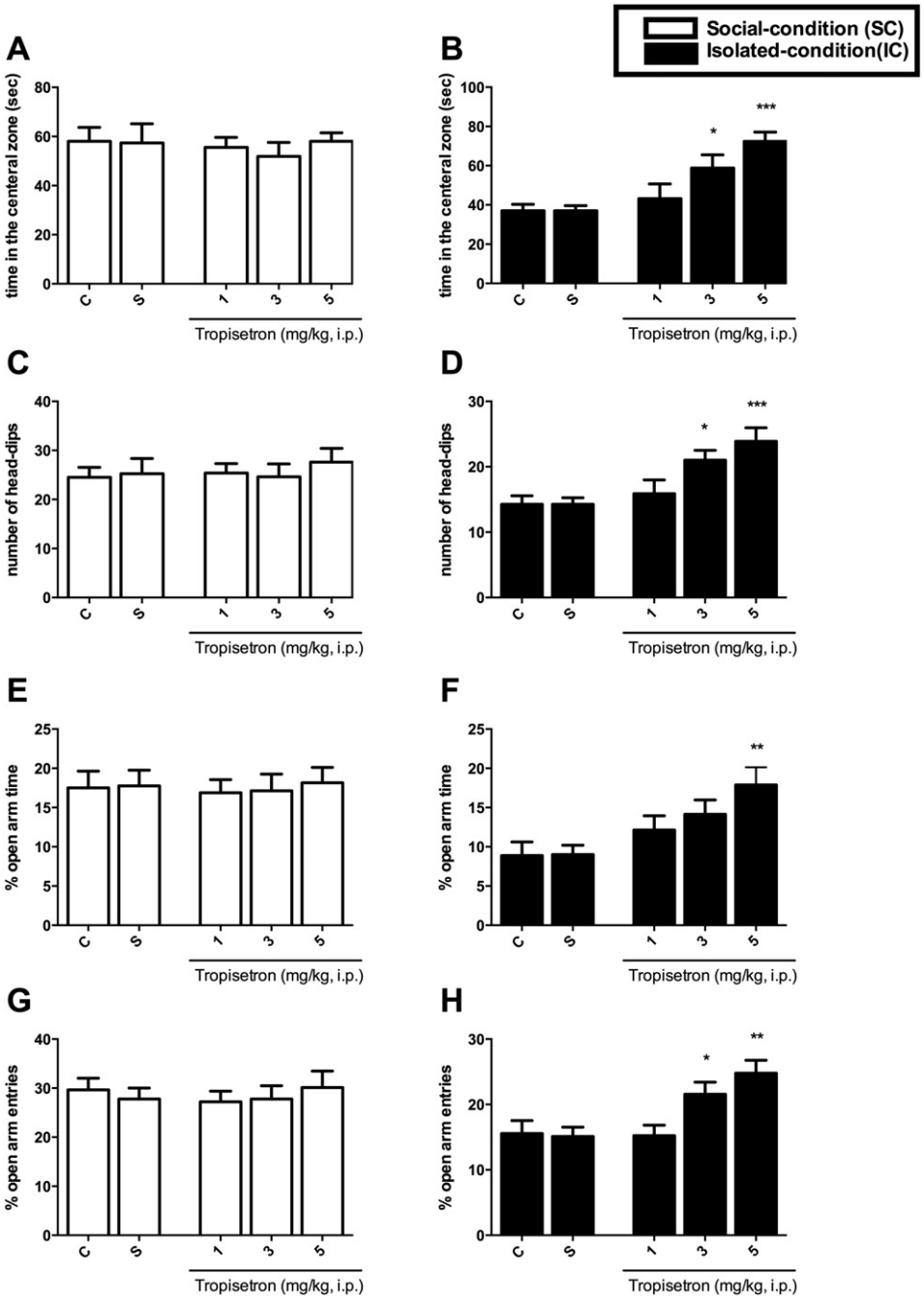

Fig. 2. Effect of tropisetron (1, 3, and 5 mg/kg) treatment on the time spent in the central zone (A, B), number of head-dips (C, D), the percentage of time spent in the open arm (E, F), and the percentage of entries in the open arm (G, H) in SC and IC animals. Values are expressed as the mean ± S.E.M from 6 to 9 animals and were analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the saline-treated IC mice (S).

ANOVA showed that there was no significant differences between treated SC animals in the OFT ($F_{(6, 35)} = 0.02005$, $P > 0.05$, Fig. 3A), HBT ($F_{(6, 49)} = 0.07436$, $P > 0.05$, Fig. 3C), and in the EPM ($F_{(6, 49)} = 0.08503$, $P > 0.05$, Fig. 3E) ($F_{(6, 56)} = 0.1118$, $P > 0.05$, Fig. 3G). However, the results were different in the IC groups; One-way ANOVA showed that there were significant differences between treated IC groups in the OFT ($F_{(6, 35)} = 5.660$, $P < 0.001$, Fig. 3B), HBT ($F_{(6, 49)} = 10.07$, $P < 0.0001$, Fig. 3D), and EPM ($F_{(6, 49)} = 4.42$, $P < 0.01$, Fig. 3F) ($F_{(6, 56)} = 2.971$, $P < 0.05$, Fig. 3H).

Tukey's analyses showed that results obtained from the OFT showed that administration of L-NAME (10 mg/kg, but not 5 mg/kg) ($P < 0.05$) and AG (50 mg/kg, but not 20 mg/kg) ($P < 0.01$) significantly increased the time spent in the central zone in the IC mice in comparison with saline-treated IC mice, while treatment with L-Arg (50 and 100 mg/kg) had no effect on the time spent in the central zone ($P > 0.05$). In the HBT as shown in Fig. 3D, subeffective doses of L-NAME (10 mg/kg, but not 5 mg/kg) ($P < 0.01$) and AG (50 mg/kg, but not 20 mg/kg) ($P < 0.001$) increased the number of head-dips when compared to saline-treated controls. However, treatment with L-arg (50 and 100 mg/kg) had no effect on the number of head-dips in the IC mice ($P > 0.05$). In comparison with saline-treated IC controls, results from EPM revealed that applying L-NAME (10 mg/kg, but not 5 mg/kg) ($P < 0.05$) and AG (50 mg/kg, but not 20 mg/kg) ($P < 0.01$) increased the percentage of time spent in the open arms, while administration of

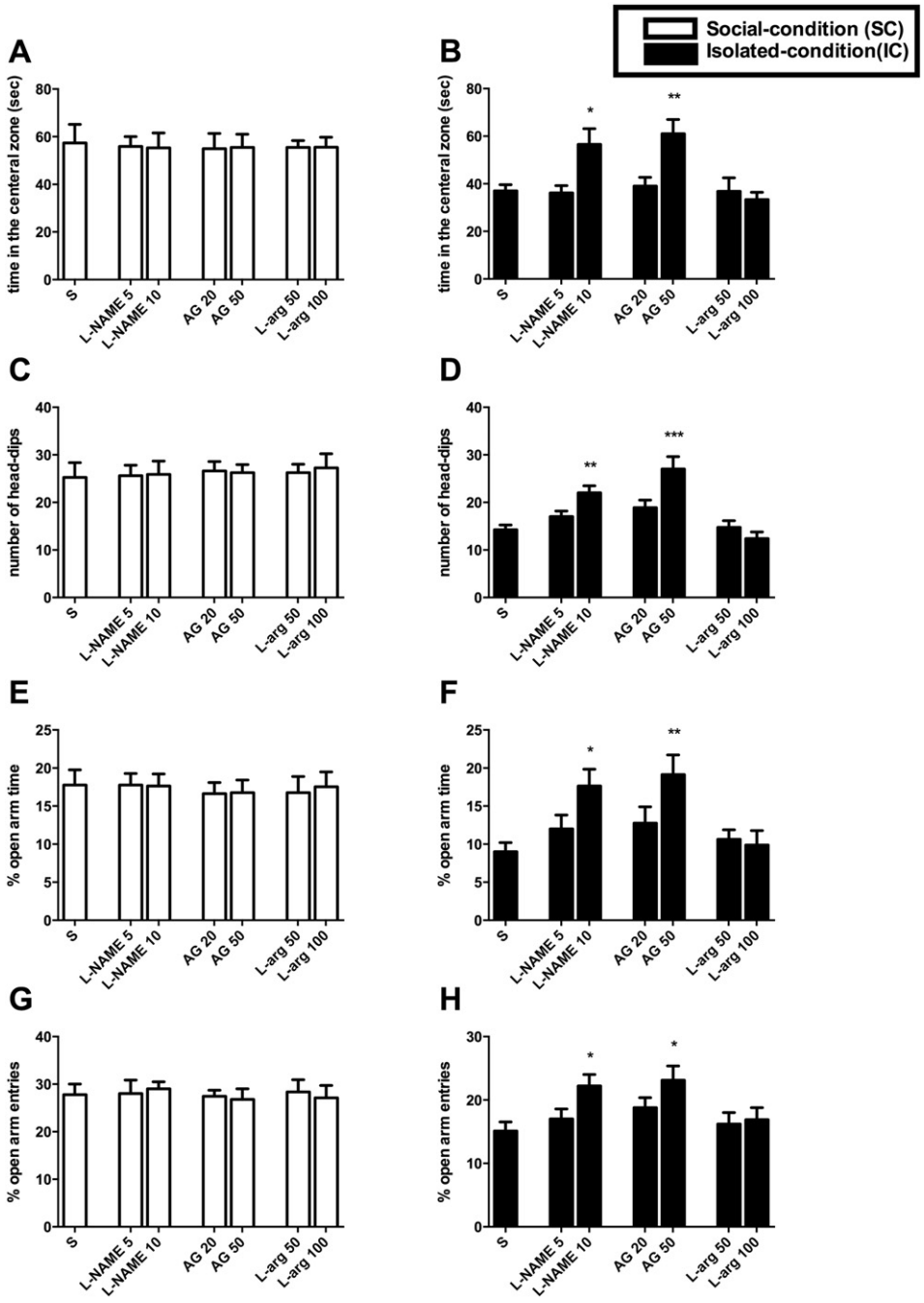

Fig. 3. Effects of NO agents including L-NAME 5 and 10 mg/kg, Aminoguanidine (AG) 20 and 100 mg/kg, and L-arginine (L-arg) 50 and 100 mg/kg on the time spent in the central zone (A, B), number of head-dips (C, D), the percentage of time spent in the open arm (E, F), and the percentage of entries in the open arm (G, H) in SC and IC animals. Values are expressed as the mean ± S.E.M from 6 to 9 animals and were analyzed using one-way ANOVA followed by Tukey's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the saline-treated IC mice (S).

L-arg (50 and 100 mg/kg) produced no significant change in latter parameters ($P > 0.05$). In addition, same as the last parts, treatment with L-NAME (10 mg/kg, but not 5 mg/kg) ($P < 0.05$) and AG (50 mg/kg, but not 20 mg/kg) ($P < 0.05$) showed significant changes in the percentage of entries in the open arms of EPM in IC animals when compared with saline treated IC mice. Administration of L-arg (50 and 100 mg/kg) produced no significant change in the percentage of entries in the open arms of EPM in IC animals ($P > 0.05$). In comparison to saline-treated SC mice, Tukey's analyses showed that administration of the same doses of above drugs had no effect on the anxiety-like behaviors of SC mice in the OFT, HBT, and in the EPM ($P > 0.05$). It is important to note that AG is a relatively specific inhibitor of iNOS and at higher concentrations; it may still suppress constitutive NOS. Thus, dosages of AG (20 and 50 mg/kg) were selected based on the previous studies that reported these dosages have no effect on constitutive NOS [43,44].

3.4. Effects of nitrergic system on anxiolytic properties of tropisetron

3.4.1. Anxiety-like behaviors in the OFT

Two-way ANOVA analysis revealed that co-administration of subeffective doses of NOS inhibitors/NO precursor with subeffective dose of tropisetron had no effect on the spent time in the central zone in comparison to saline-treated SC mice ($P > 0.05$, Fig. 4A, C, E).

On the other hand, co-administration of L-NAME (5 mg/kg)along with tropisetron (1 mg/kg) increased the spent time in the central zone in IC animals when compared to saline-treated IC controls ($P < 0.05$, Fig, 4B). A two way ANOVA revealed significant differences in the OFT for the tropisetron treatment ($F_{(1, 20)} = 11.08$, $P < 0.01$), L-NAME treatment ($F_{(1, 20)} = 5.610$, $P < 0.05$), and their interaction ($F_{(1, 20)} = 4.365$, $P < 0.05$).

Also, injecting a subeffective dose of AG (20 mg/kg) to tropisetron-treated IC animals (1 mg/kg) caused an obvious anxiolytic effect when

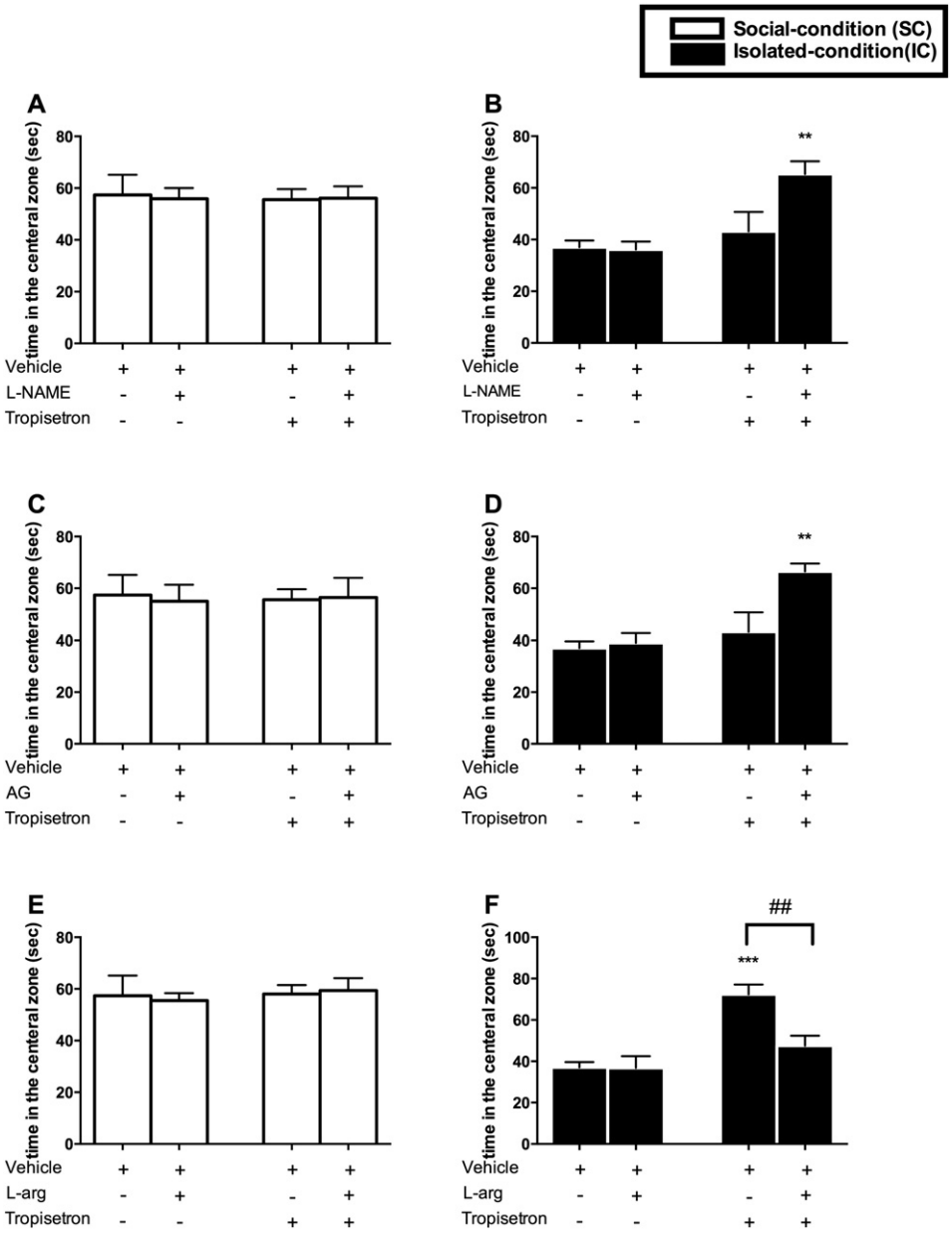

Fig. 4. Effect of L-NAME pretreatment (5 mg/kg) on the tropisetron-treated animals (1 mg/kg) in SC and IC animals (A, B), Effect of pretreatment with AG (20 mg/kg) on subeffective tropisetron-treated animals (1 mg/kg) in SC and IC animals (C, D) and, Effect of pre-treatment with L-arg (50 mg/kg) on tropisetron-treated animals (5 mg/kg) (E, F) in SC and IC animals. Animals were tested using OFT and time spent in the central zone was evaluated. Values are expressed as the mean ± S.E.M. (n = 6–9) using two-way ANOVA. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with vehicle-treated group. $^{\#\#}P < 0.01$ compared with tropisetron (5 mg/kg)-treated group.

compared to saline-treated IC mice ($P < 0.01$, Fig. 4D). A two way ANOVA revealed significant differences for the tropisetron treatment ($F_{(1, 20)} = 13.11$, $P < 0.01$), AG treatment ($F_{(1, 20)} = 7.479$, $P < 0.05$), and their interaction ($F_{(1, 20)} = 5.253$, $P < 0.05$).

In addition, treatment with L-arg (50 mg/kg) abolished the anxiolytic effect of tropisetron 5 mg/kg via decreasing the spent time in the central zone when compared to IC saline group ($P < 0.01$, Fig. 4F). A two way ANOVA revealed significant differences for the tropisetron treatment ($F_{(1, 20)} = 25.04$, $P < 0.0001$), L-arg treatment ($F_{(1, 20)} = 7.345$, $P < 0.05$), and tropisetron × L-arg ($F_{(1, 20)} = 7.150$, $P < 0.05$).

3.4.2. Anxiety-like behaviors in the HBT

Co-administration of L-NAME (5 mg/kg) with tropisetron (1 mg/kg) increased the number of head-dips in the IC animals in comparison with saline-treated IC mice ($P < 0.05$, Fig. 5B). A two way ANOVA revealed significant differences in the HBT for the tropisetron treatment ($F_{(1, 28)} = 4.233$, $P < 0.05$) and L-NAME treatment ($F_{(1, 28)} = 8.003$, $P < 0.01$), but not their interaction ($F_{(1, 28)} = 0.8892$, $P < 0.05$).

On the other hand, combination of the subeffective doses of the tropisetron with AG (20 mg/kg) significantly affected the number of head-dips of IC mice ($P < 0.05$, Fig. 5D) and in two-way ANOVA we observed significant differences between tropisetron treatment ($F_{(1, 28)} = 10.36$, $P < 0.01$), AG treatment ($F_{(1, 28)} = 23.30$, $P < 0.0001$), and tropisetron × AG ($F_{(1, 28)} = 5.506$, $P < 0.05$).

In comparison with saline-treated IC mice, co-administration of L-arg (50 mg/kg) with tropisetron (5 mg/kg) impaired the anxiolytic-like effect of tropisetron by decreasing the number of head-dips ($P < 0.05$, Fig. 5D). Also, two way ANOVA showed significant differences for the tropisetron treatment ($F_{(1, 28)} = 9.925$, $P < 0.01$), L-arg treatment ($F_{(1, 28)} = 4.311$, $P < 0.05$), and tropisetron × L-arg treatment

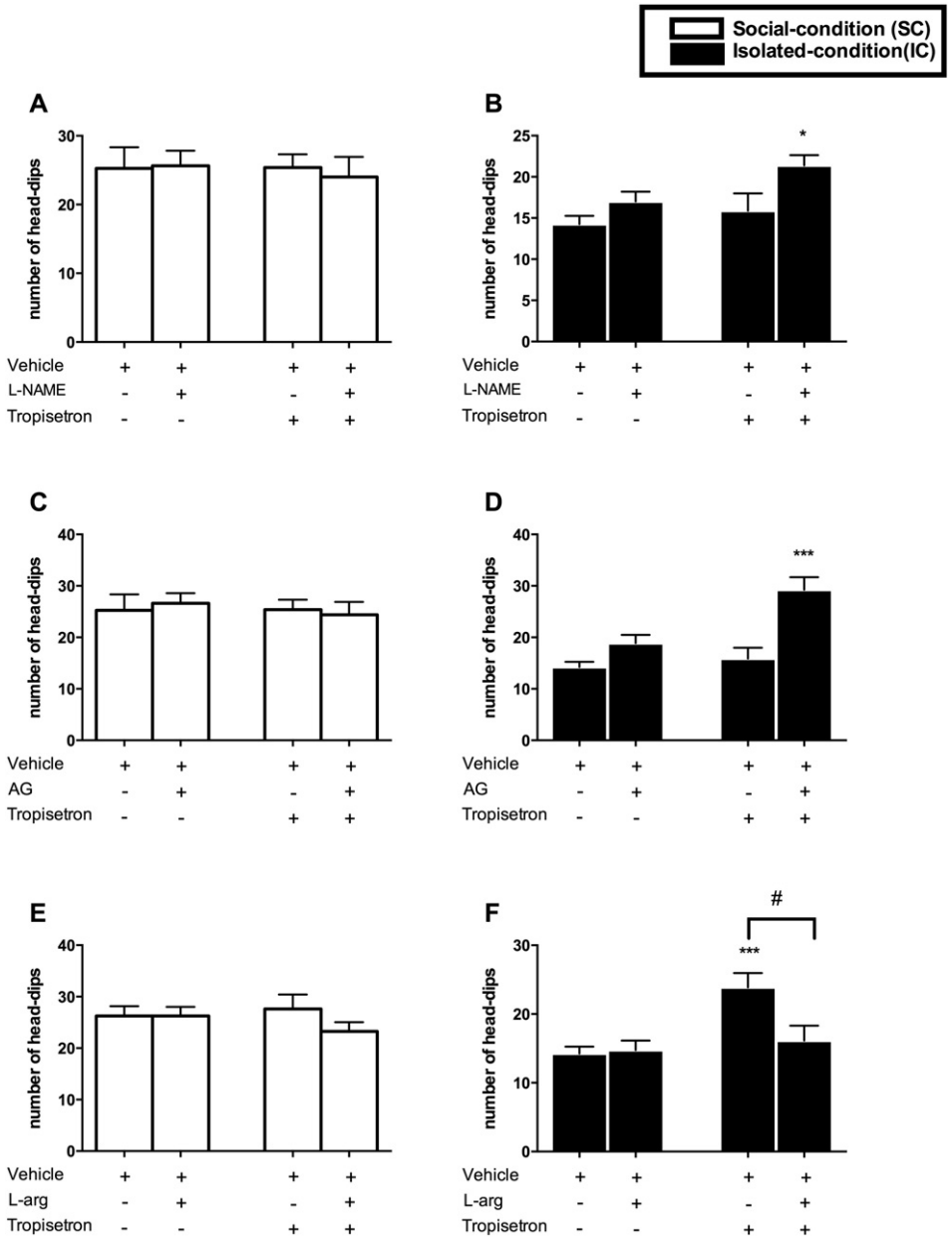

Fig. 5. Effect of L-NAME pretreatment (5 mg/kg) on the tropisetron-treated animals (1 mg/kg) in SC and IC animals (A, B), Effect of pretreatment with AG (20 mg/kg) on subeffective tropisetron-treated animals (1 mg/kg) in SC and IC animals (C, D) and, Effect of pre-treatment with L-arg (50 mg/kg) on tropisetron-treated animals (5 mg/kg) (E, F) in SC and IC animals. Animals were tested using HBT and number of head-dips was measured. Values are expressed as the mean ± S.E.M. (n = 6–9) using two-way ANOVA. *$P < 0.05$ and***$P < 0.001$ compared with vehicle-treated group. #$P < 0.05$ compared with tropisetron (5 mg/kg)-treated group.

($F_{(1, 28)} = 5.583$, $P < 0.05$). In comparison to saline-treated SC mice, administration of the same doses of above drug combinations had no effect on the anxiety-like behaviors of SC mice in HBT ($P > 0.05$, Fig. 5A, C, E).

3.4.3. Anxiety-like behaviors in the EPM

In comparison to saline-treated controls, treatment the IC mice with combination of tropisetron (1 mg/kg) and L-NAME (5 mg/kg) significantly increased the percentage of time spent in the open arms ($P < 0.05$, Fig. 6B) as well as percentage of open arms entries ($P < 0.01$, Fig. 7B). Also, two way ANOVA analysis demonstrated significant differences in the percentage of time spent in the open arms and open arms entries for the tropisetron treatment ($F_{(1, 28)} = 5.628$, $P < 0.05$; $F_{(1, 32)} = 4.235$, $P < 0.05$) and L-NAME treatment ($F_{(1, 28)} = 5.287$, $P < 0.05$; $F_{(1, 32)} = 10.32$, $P < 0.01$), but not tropisetron × L-NAME treatment ($F_{(1, 28)} = 0.2997$, $P > 0.05$; $F_{(1, 32)} = 3.943$, $P > 0.05$).

In addition, co-administration of tropisetron (1 mg/kg) with AG (20 mg/kg) induced a significant increase in the percentage of time spent in the open arms ($P < 0.01$, Fig. 6D) as well as percentage of open arms entries ($P < 0.001$, Fig. 7D) as compared to saline-treated IC mice. A two way ANOVA showed mostly significant differences in the percentage of time spent in the open arms and open arms entries for the tropisetron treatment ($F_{(1, 28)} = 7.152$, $P < 0.01$; $F_{(1, 32)} = 5.378$, $P < 0.05$), AG treatment ($F_{(1, 28)} = 9.276$, $P < 0.05$; $F_{(1, 32)} = 20.86$, $P < 0.0001$), and tropisetron × AG treatment ($F_{(1, 28)} = 0.6677$, $P > 0.05$; $F_{(1, 32)} = 5.057$, $P < 0.05$).

Also, comparison with saline-treated IC controls showed that administration of L-arg (50 mg/kg) abolished the anxiolytic-like effects of tropisetron (5 mg/kg) by reducing the percentage of spent time in the open arms ($P < 0.01$, Fig. 6F) as well as percentage of open arms entries ($P < 0.001$, Fig. 7F). Two way ANOVA analysis revealed significant differences in the percentage of time spent in the open arms and open

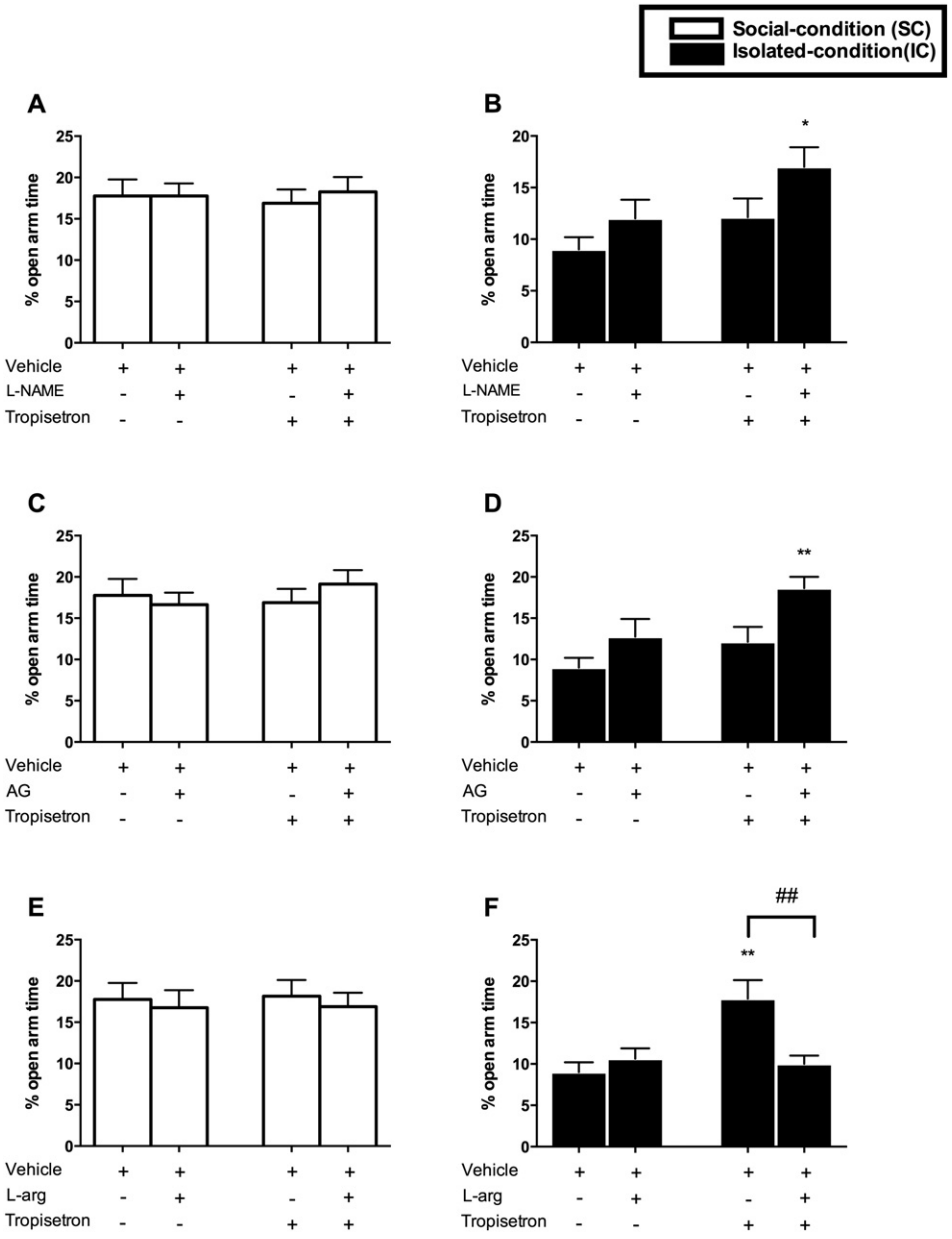

Fig. 6. Effect of L-NAME pretreatment (5 mg/kg) on the tropisetron-treated animals (1 mg/kg) in SC and IC animals (A, B), Effect of pretreatment with AG (20 mg/kg) on subeffective tropisetron-treated animals (1 mg/kg) in SC and IC animals (C, D) and, Effect of pre-treatment with L-arg (50 mg/kg) on tropisetron-treated animals (5 mg/kg) (E, F) in SC and IC animals. Animals were tested using EPM and the percentage of time spent in the open arm was measured. Values are expressed as the mean ± S.E.M. (n = 6–9) using two-way ANOVA. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with vehicle-treated group. $\#\#P < 0.01$ compared with tropisetron (5 mg/kg)-treated group.

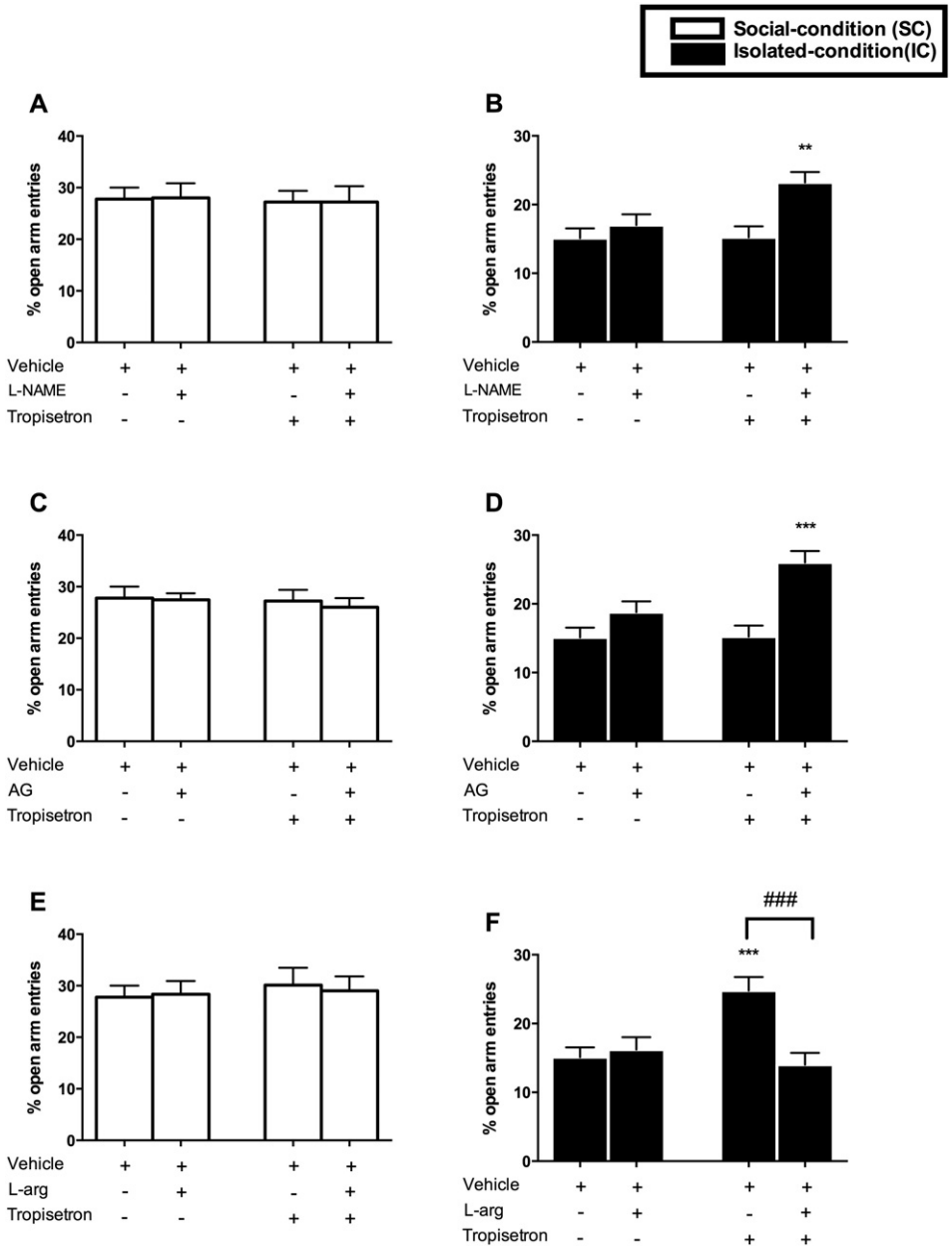

Fig. 7. Effect of L-NAME pretreatment (5 mg/kg) on the tropisetron-treated animals (1 mg/kg) in SC and IC animals (A, B), Effect of pretreatment with AG (20 mg/kg) on subeffective tropisetron-treated animals (1 mg/kg) in SC and IC animals (C, D) and, Effect of pre-treatment with L-arg (50 mg/kg) on tropisetron-treated animals (5 mg/kg) (E, F) in SC and IC animals. Animals were tested using EPM and the percentage of entries in the open arm was measured. Values are expressed as the mean ± S.E.M. (n = 6–9) using two-way ANOVA. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with vehicle-treated group. $^{###}P < 0.001$ compared with tropisetron (5 mg/kg)-treated group.

arms entries for the tropisetron treatment ($F_{(1, 28)} = 7.433$, $P < 0.05$; $F_{(1, 32)} = 4.504$, $P < 0.05$), L-arg treatment ($F_{(1, 28)} = 4.266$, $P < 0.05$; $F_{(1, 32)} = 7.594$, $P < 0.01$), and tropisetron × L-arg treatment ($F_{(1, 28)} = 9.857$, $P < 0.01$; $F_{(1, 32)} = 11.49$, $P < 0.01$). In comparison to saline-treated SC mice, administration of the same doses of above drug combinations had no effect on the anxiety-like behaviors of SC mice in any of behavioral assessments in the EPM ($P > 0.05$, Fig. 6A, C, E and Fig. 7A, C, E).

3.5. Effects of different housing conditions and drug treatments on mitochondrial function and O&NS

3.5.1. Effects of housing conditions and drug treatments on GSH levels in the hippocampus

Table 1 shows a significant decrease in GSH levels of hippocampal cytosolic fraction of IC mice in comparison with SC animals using Tukey's analysis ($P < 0.01$). Administration of tropisetron (5 mg/kg) significantly restored GSH levels in comparison with saline-treated IC mice ($P < 0.001$). However, However, Tukey's analysis showed that treatment with tropisetron 1 mg/kg failed to induce any significant change in GSH levels when compared to saline-treated IC mice ($P > 0.05$). Similarly, administration of subeffective doses of L-NAME (5 mg/kg), AG (20 mg/kg), and L-arg (50 and 100 mg/kg) had no significant change on the hippocampal GSH levels in IC mice as compared to saline controls ($P > 0.05$). However, higher doses of L-NAME (10 mg/kg) ($P < 0.05$) and AG (50 mg/kg) ($P < 0.01$) significantly affect the hippocampal GSH levels in IC animals. In comparison with saline treated IC mice, treating IC mice with AG 20 mg/kg (but not L-NAME 5 mg/kg) in combination with tropisetron (1 mg/kg) significantly restored GSH levels ($P < 0.001$). In addition, administration of L-arg (50 mg/kg) significantly lowered the elevated levels of GSH in IC mice treated with tropisetron 5 mg/kg ($P < 0.001$).

Table 1
Effect of different housing conditions and treatments on glutathione (GSH), ATP, and nitrite level in hippocampus.

Groups	GSH (μg/mg protein)	ATP (nmol/mg protein)	Nitrite (nmol/mg protein)
Social condition (SC)	14.7 ± 2.3	3.1 ± 0.1	81 ± 5
Isolated condition (IC)	7.1 ± 0.8**	1.9 ± 0.1**	143 ± 6***
+ Tropisetron (1 mg/kg)	8.1 ± 0.8**	1.9 ± 0.3**	133 ± 5***
+ Tropisetron (5 mg/kg)	14.7 ± 2.6 ###	3 ± 0.5###	93 ± 5##
+ L-NAME (5 mg/kg)	7.6 ± 1.1**	1.8 ± 0.4**	137 ± 6***
+ L-NAME (10 mg/kg)	9.8 ± 0.6#	2.6 ± 0.3#	112 ± 3#
+ Aminoguanidine (20 mg/kg)	7.8 ± 1**	1.9 ± 0.2**	135 ± 8***
+ Aminoguanidine (50 mg/kg)	11.6 ± 1.2##	3 ± 0.6##	120 ± 5**
+ L-arginine (50 mg/kg)	7 ± 1.3**	1.8 ± 0.3**	147 ± 9***
+ L-arginine (100 mg/kg)	6.8 ± 0.9**	1.9 ± 0.4**	157 ± 11***
+ Tropisetron (1 mg/kg) + L-NAME (5 mg/kg)	7.6 ± 1.6**	2.4 ± 0.1 #	109 ± 8#
+ Tropisetron (1 mg/kg) + Aminoguanidine (20 mg/kg)	11.3 ± 2.5###	3.1 ± 0.3 ###	78 ± 6###
+ Tropisetron (5 mg/kg) + L-Arginine (50 mg/kg)	7.4 ± 1.3***	1.8 ± 0.2**	129 ± 7***

Reduced GSH levels were determined according to the method of Riener et al. (2002) using Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid, DTNB) as described in Materials and methods.ATP level were determined using Luciferin/Luciferase Enzyme System as described in Materials and methods. Nitrite level was evaluated based on our previously described method. Values represented as mean ± S.E.M. (n = 3–6). *P < 0.05, **P < 0.01, and ***P < 0.001 compared with SC group and #P < 0.05; ##P < 0.01; ###P < 0.001 compared with IC group.

3.5.2. Effects of housing conditions and drug treatments on ATP levels in the hippocampus

Results obtained from Tukey's analysis revealed that there is a significant decrease in ATP production in the hippocampus of IC mice as compared with SC controls ($P < 0.01$). Also, administration of subeffective doses of AG (20 mg/kg), L-NAME (5 mg/kg), L-arg (50 and 100 mg/kg), and tropisetron (1 mg/kg) did not restore ATP levels in the hippocampus of IC animals when compared to saline-treated IC mice ($P > 0.05$). Tukey's analysis demonstrated that treatment of IC mice with tropisetron (5 mg/kg) increased ATP levels in the hippocampus in comparison with saline-treated IC mice ($P < 0.001$). Furthermore, co-administration of subeffective dose of L-arg (50 mg/kg) abolished the effect of tropisetron on ATP generation ($P < 0.01$). On the other hand, co-administration of L-NAME (5 mg/kg) or AG (20 mg/kg) with tropisetron (1 mg/kg) significantly increased ATP levels in the hippocampus of IC mice in comparison with IC controls ($P < 0.05$ and $P < 0.001$ respectively).

Table 2
% Increased of ROS formation in hippocampus after treatment with different drugs in social and isolated condition.

Groups	DCF fluorescence intensity (%)	
	5 min	45 min
Social condition (SC)	2 ± 1	8 ± 3
Isolated condition (IC)	15 ± 3**	187 ± 16***
+ Tropisetron (1 mg/kg)	21 ± 1***	147 ± 5***
+ Tropisetron (5 mg/kg)	4 ± 2###	38 ± 3 ###
+ L-NAME (5 mg/kg)	16 ± 5***	163 ± 21***
+ L-NAME (10 mg/kg)	9 ± 2#	79 ± 16##
+ Aminoguanidine (20 mg/kg)	14 ± 3**	151 ± 19***
+ Aminoguanidine (50 mg/kg)	6 ± 2###	45 ± 13###
+ L-arginine (50 mg/kg)	16 ± 4**	184 ± 21***
+ L-arginine (100 mg/kg)	17 ± 3**	199 ± 23***
+ Tropisetron (1 mg/kg) + L-NAME (5 mg/kg)	9 ± 2##	37 ± 9###
+ Tropisetron (1 mg/kg) + aminoguanidine (20 mg/kg)	5 ± 1###	15 ± 2 ###
+ Tropisetron (5 mg/kg) + L-arginine (50 mg/kg)	11 ± 2*	21 ± 4***

ROS generation measured by DCF formation method expressed as fluorescent intensity units as described in Materials and methods and demonstrated as fluorescence intensity of DCF. Values represented as mean ± S.E.M. (n = 3–6). Social condition (SC; Control) group contains respiration buffer plus DCFH-DA (10 μM). *P < 0.05; **P < 0.01; ***P < 0.001 compared with SC group and #P < 0.05; ##P < 0.01; ###P < 0.001 compared with IC group at the same time interval. Fluorescence intensity (%) is described as the percentage of fluorescence intensity compared with social condition (SC) groups.

3.5.3. Effects of housing conditions and drug treatments on nitrite levels in the hippocampus

Tukey's analysis revealed that IC mice have greater levels of nitrite content in comparison with SC animals ($P < 0.001$). Also, there was no significant difference in the hippocampal nitrite levels between saline-treated IC mice and IC mice treated with subeffective doses of AG (20 and 50 mg/kg), L-NAME (5 mg/kg), L-arg (50 and 100 mg/kg), and tropisetron (1 mg/kg) ($P > 0.05$). In comparison with saline-treated IC mice, a significant decrease in hippocampal nitrite levels was observed, when IC mice were treated with tropisetron (5 mg/kg) ($P < 0.01$). Also, co-administration of subeffective doses of L-arg (50 mg/kg) blocked the effect of tropisetron on nitrite reduction ($P < 0.001$). Furthermore, co-administration of L-NAME (5 mg/kg) or AG (20 mg/kg) with tropisetron (1 mg/kg) significantly decreased the nitrite levels in the hippocampus of IC mice in comparison with IC controls ($P < 0.05$ and $P < 0.001$ respectively) (Table 2).

3.5.4. Effects of housing conditions and drug treatments on ROS formation in the hippocampus

Assessment of ROS formation performed in 2 time intervals (5 min and 45 min) as described above. A sizable increase in hippocampal ROS generation was observed in IC mice at both 5 and 45 min intervals when compared to SC controls using tukey's analysis ($P < 0.01$ and $P < 0.001$ respectively). Treating IC mice with AG (20 mg/kg), L-NAME (5 mg/kg), L-arg (50 and 100 mg/kg), and tropisetron (1 mg/kg) did not affect the elevated levels of ROS formation in the hippocampus when compared with saline-treated IC animals ($P > 0.05$). Also, in comparison with IC controls, administration of tropisetron (5 mg/kg) to IC animals reversed the hippocampal ROS levels to normal state ($P < 0.001$), and the effect was blocked by administration of L-arg (50 mg/kg) ($P < 0.05$ and $P < 0.001$). Moreover, co-administration of L-NAME (5 mg/kg) or AG (20 mg/kg) with tropisetron (1 mg/kg) decreased the enhanced ROS production in the hippocampus of IC animals as compared to IC controls at both 5 min ($P < 0.01$ and $P < 0.001$ respectively) and 45 min ($P < 0.001$ and $P < 0.001$ respectively) intervals (Table 2).

4. Discussion

Results of the current study revealed that experiencing early SIS during developmental stages of brain in the adolescence produces anxiety-like behaviors and impairs mitochondrial activity in the hippocampus of animals. Our results also provided preliminary evidence that tropisetron exerts anxiolytic effect via modulating the NO, which per se mediates

the anxiogenic effects of SIS by enhancing the O&NS through mitochondrial challenge.

Applying animal models of chronic stress provide conditions to investigate underlying mechanisms of stress-related behaviors similar to anxiety disorders in humans [31]. Previous studies have reported that SIS is a cogent psychosocial stress, which capable of inducing emotional changes as well as enduring and permanent anxious state in rodents [45]. In the current study, we evaluated the anxiety-like responses of adult mice using behavioral tests, which were approved as valid paradigms to assess anxiety-like behaviors including EPM, OFT, and HBT. Applying SIS to adolescent mice induced significant anxiogenic effects, which reversed by administration of subeffective doses of tropisetron, L-NAME, and AG. Also, co-administration of subeffective doses of AG-tropisetron and L-NAME-tropisetron produced the same effects. However, these treatments had no effect on SC mice. Stress induces a variety of neurochemical changes in the brain that leads to elevation of excitatory amino acids such as glutamate, which causes an overproduction of NO in the brain [46,47]. Increased expression of NOS isoforms or elevated levels of NO metabolites was reported in the limbic areas in various studies using SIS paradigm [48,49]. Also, participation of NO in anxiety-like behaviors after stressful conditions is well documented in the literature. Similar to our results, there are studies reported that administration of L-NAME and AG exhibited anxiolytic properties in rodents [17,50,51].

Evidence indicated that early SIS disrupts the maturation of neurotransmission systems such as serotonergic system that regulates anxiety-like behaviors. Alterations in the serotonergic system following early SIS have been reported in different regions of the brain including limbic area [6]. Limbic system is considered to be involved in the anxiety-like behaviors and has a rich content of 5-HT3 receptors. These receptors strongly contribute to maturation of inhibitory networks that moderate the excitatory neurotransmission. Also, 5-HT3 receptors are implicated in pathophysiology of the several developmental disorders including affective disorders [23]. Our results were consistent with previous studies that reported 5-HT3 receptor antagonists, in addition to their anxiolytic properties, are able to decrease the NO levels as well as iNOS expression in the brain under pathologic conditions [52]. Therefore, protective effect of tropisetron against O&NS as well as behavioral alterations in IC mice may be related to modulate the nitrergic system (mainly iNOS) in the brain. In this context, our results revealed that anxiolytic effects of tropisetron in the EPM (increase the percentage of entries and spent time in the open arms), OFT (increase in the spent time in central zone) and HBT (increase in number of head-dips) are mediated partly by nitrergic system. To support this, we measured the levels of ROS generation, GSH, nitrite and ATP in the hippocampus of experimental groups. Results showed that SIS negatively upsets mitochondrial function by massive production of ROS and nitrite as well as a significant decrease in ATP and GSH levels. Ample evidence indicates that developing brain is specifically susceptible to O&NS–mediated damage because of a high contents of unsaturated fatty acids, low levels of antioxidant capacity and also high rate of oxygen consumption [53]. Our findings showed that co-administration of subeffective doses of tropisetron and NOS inhibitors (L-NAME, AG) or treatment with tropisetron alone effectively succeeded to reverse the elevated levels of ROS and nitrite as well as GSH and ATP depletion in the hippocampus of IC mice. In this respect, best results were observed with co-administration of the tropisetron and AG, which potently improved the oxidative challenge in IC mice. It is important to note that unlike IC mice, same treatments had no effect on the hippocampal nitrite, ATP, ROS and GSH levels in SC mice. Additionally, L-arg as a NO precursor blocked the protective effects of latter treatments against SIS-induced O&NS in IC and not SC mice. Accumulating evidence suggests that oxidative stress and antioxidant imbalance contribute to pathogenesis of mood and anxiety disorders [8–10]. Evidence reported that psychosocial stressors cause impairments in the oxidant/antioxidant equilibrium through boosting the generation of ROS and NO in the specific brain regions such as hippocampus [54]. Also, previous research has shown that psychosocial stressors decrease GSH levels in the brain which is accompanied by reduced activity of antioxidant enzymes and mitochondrial dysfunction [15,55]. Based on our results, SIS-induced changes in mitochondrial function and O&NS were accompanied by anxiety-like behaviors in IC mice. In this case, Filiou et al. revealed that the mouse model of high anxiety-related behavior has mitochondrial dysfunction in the brain. They also showed that mitochondrial dysfunction plays a part in neurotransmission disequilibrium which has been considered as the hallmark of anxiety disorders [7,56]. In addition, findings of other laboratories corroborated the involvement of mitochondrial dysfunction in etiology of psychiatric disorders including depression [57,58]. Also, clinical studies revealed that mood disorders are of most prevalent symptoms observed in children and adolescents with mitochondrial disorders [59,60]. In conclusion, results of this study showed that anxiogenic effect of early SIS was partly mediated by NO-induced O&NS along with impaired mitochondrial function. Also, our findings revealed that nitrergic system plays a role in anxiolytic properties of tropisetron in IC mice via reversing the negative impact of SIS on mitochondrial performance.

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