Heliyon 10 (2024) e28964

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

The role of gender differences in the outcome of juvenile social isolation: Emphasis on changes in behavioral, biochemical and expression of nitric oxide synthase genes alteration

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ARTICLE INFO

Keywords:
Post-weaning social isolation (PWSI)
Gender difference
Depression
Oxidative stress
Nitric oxide synthase (NOS)
Gene expression

ABSTRACT

Social isolation can cause serious problem in performance of individuals in community. As gender differences may cause variation results in the severity of depressive behavior and response of patients to therapy, the impact of gender and the interaction of the level of endocrine secretion in depression were investigated in this study. Wistar rats of both sexes were subjected to post-weaning social isolation (PWSI) conditions and, together with the control group, experienced several behavioral tests including open-field Test (OFT), elevated plus maze (EPM), force swimming test (FST), splash test and novel object recognition test (NOR). Hippocampal tissue was isolated to measure biochemical factors such as nitric oxide level, FRAP amount, MDA level. In addition, real-time-PCR test was used to quantify the genes expression level of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS). On the other hand, sexual hormone levels in blood were measured. Both cognitive and behavioral f unctions were declined as the result of PWSI induction in male and diestrus female rats. The consequent surge of estradiol during estrous phase seems to suppress the accumulation of reactive oxygen species (ROS), and modulate iNOS and nNOS expression. In conclusion, while the pattern of PWSI in surge cellular antioxidants, raising cellular ROS level is gender-specific, this alleviation was in relation with the drop of estradiol and unrelated with testosterone level.

1. Introduction

Depression is one of the most common physiological disorders in the world. In Europe and America, it is estimated that 5–12% of men and 9–26% of women suffer from depressive disorders. Welfare and social conditions can affect the prevalence of this disorder [1]. In the last two decades, the rate of family foundation has decreased by 10%. While a family has an average of 2.5 people, the number of one-person families has increased by 30%. Social isolation has effect on our life expectancy and well-being. This disorder can cause disrupted sleep patterns, depression, and apathetic behavior [2]. Although the distribution of monoamine secretion is recognized as a

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https://doi.org/10.1016/j.heliyon.2024.e28964
Received 21 July 2023; Received in revised form 27 March 2024; Accepted 27 March 2024
Available online 1 April 2024

Abbreviations

PWSI	Post-weaning social isolation
NOR	novel object recognition
NOS	Nitric oxide synthase
OFT	Open-Field Test
EPM	Elevated plus maze
FST	Forced swimming test
NO	Nitric oxide
FRAP	Ferric reducing antioxidant power
LPO	Lipid peroxidation

central aspect of the biochemistry of depression [3], new studies are investigating factors such as the endocrine system and neurotrophic factors to demonstrate their role in the pathophysiology of depression caused by isolation [4,5]. It seems that the hypothalamic-pituitary-adrenal (HPA) axis has a significant effect on the pathophysiology of depressive disorder. On the other hand, the secretion of sex hormones is one of the important aspects of HPA axis activity [6]. Animal models play a crucial role in studying social isolation, since they mimic a closely sign and symptoms of cognitive and neurological aspects of this disease, namely depression, anxiety and memory loss, and tend to represent biochemical alternations similar to humans [7].

Hypersensitivity to emotional stress leads to neuro-inflammation, which can cause excessive accumulation of reactive oxygen species (ROS) and relative neurodegeneration in the hippocampus [8,9]. Apart from measuring direct level of ROS content, the oxidized lipids and peroxidized proteins can also be measured as byproducts of cellular ROS content [10].

One of the areas effected by oxidative stress is mitigation of neurotrophic factors, which play an important role in the development of the nervous system, maintaining neural plasticity and creating new neural connections [11]. Chronic pain and anxiety can reduce the secretion of neurotropic factors, which can lead to a 10–15% decrease in hippocampal volume. SSRI treatment can lead to a significant improvement in the levels of these factors, especially BDNF [1,12].

As an intracellular messenger with a short half-life, nitric oxide plays a noticeable role in modulating various neurotransmitters such as norepinephrine, glutamate, and serotonin. In addition, some behavioral patterns such as feeling pain, aggressive behavior and learning can also be affected by changing NO levels [13]. Pharmacological suppression of NO synthesis has led to an increase in response to stimuli, induction of anxiogenic behavior and decrease in expression of neurotrophic factors in animals [14]. It has been shown that paroxetine as selective serotonin reuptake inhibitor, can reduce NOS activity and caused anxiolytic effects [15]. Decrease in NOS activity due to the role of NO in clot formation can lead to coronary heart disease. Antidepressants such as milnacipran can be used in these patients to the increase the level of NO production [16]. In this study, we used wistar rat model for PWSI in order to understand the role of gender difference in severity of cognitive, biochemical, sexual hormones and histopathological alternations. We expect that during the rise of sexual hormones, due to the effect of these substances in activity of HPA-axis, the severity of malfunctions ameliorates.

2. Materials and methods

2.1. Animals

48 male and 96 female Wistar rats were kept in normal laboratory conditions with free access to food/water, day/night cycle (12:12 h), constant temperature (23 ± 2 °C) and humidity (65%). All experiments on rodents were performed according to NIH guidelines and were approved by the Animal Ethics Committee of Zanjan University of Medical Sciences (Ethical Code: ZUMS.REC. 1400.294).

2.2. Identification of estrous and diestrus cycle

The phase of the estrous cycle of female rats was determined using the previous described protocol [17]. Briefly, vaginal cells were collected using a tip filled with sterile ddH_2O and placed on a glass slide. The dried smear was stained with crystal violet and examined under the microscope for the presence of nucleated epithelial cells, cornfield squamous epithelial cells, and leukocytes. Based on the results of vaginal cytology, female rats were divided into Estrus (Proestrus, Estrus) and Diestrus (Metestrus, Diestrus) groups.

2.3. Social isolation

Male and female Wistar albino rats were purchased from the Pasteur Institute, Tehran, Iran. Following one weeks of acclimatization at the animal facility of Zanjan University of Medical Sciences. Each male rat was mated with two female rats and then, litters and their dams were housed together as family units until weaned. For social isolation, rats were separated from their mothers 21 days after birth and kept in separate polypropylene cages (15 x 25 × 25 cm) for 5 weeks. The social and isolated rats were randomly divided into 6 groups: two control groups of female rats in the estrous and diestrus phases, two groups of female rats under PWSI in the estrus and

diestrus phases, and the last two groups including male PWSI rats and normal social condition rats. In order follow the sexual pattern of female rats, vaginal samples were collected after evaluation of behavioral test in each animals [18]. Behavioral assessments were performed during the period between 10:00 and 14:00. To minimize the effects of behavioral procedures on the dependent variables of interest, three groups of animals were used for evaluation of behavioral tests (8 rodents for each group for each behavioral test): Group1 (evaluation of NOR test behavior); Group 2 (evaluate behaviors in the following order; EPM and splash test). Group 3 (evaluate behaviors in the following order; OFT and FST). Then 24h later were deeply anesthetized and euthanized by cervical dislocation.

2.4. Behavioral tests

2.4.1. Open-field test (OFT)

In order to investigate the effect of social isolation on vertical activity (locomotor activity) OFT test was performed. The maze is an opaque box made of Plexiglas (50 × 50 × 50 cm) the surface of the box was divided into 16 identical squares. After animals were separately placed in central zone their horizontal and vertical activity were analyzed in the span of 5 min [19].

2.4.2. Elevated plus maze (EPM)

EPM consists of two open arms and two covered ones designed to investigate anxiety in rodents. Anxiety would cause rodents to spend more time in the closed arms with less exposure. Their behavior was analyzed in 5 min span of the test [20].

2.4.3. Forced swimming test (FST)

FST is one of the main available tests to evaluate depression and despair behavior in rodents. For this test, glass cylinder (10 × 25 cm, diameter × height) was filled by 19 cm water level at 23 ± 1 °C. Rodents kept themselves floating on the surface of water and the time which they made no attempt to rescue was measured [21].

2.4.4. Splash test

In this test, 10% sucrose was sprayed on the nape of the rodents, animals demonstrate anhedonia by spending less time grooming and cleaning their body off sucrose solution. This grooming activity was analyzed in 5 min span [22].

2.4.5. Novel object recognition (NOR) test

NOR test is used to evaluate cognition, particularly recognition memory in rodent. Depressive behavior can decrease the discrimination ratio of NOR test. In this investigation, rodents would adapt to two novel identical objects in 15 min span for two days prior to main test, and in the main test one object is being replaced by one unfamiliar one, then rodents are left for 3 min to explore both objects. The discrimination ratio is calculated by dividing the time of exploring the novel object to the total time of test [23].

2.5. Biochemical tests

24 h after the last behavioral test, rodents were sacrificed, and their brain and blood plasma were collected. The samples were stored at −80 °C for further biochemical investigations.

2.5.1. Nitric oxide (NO) assay

Changes in Nitric oxide levels have a significant effect on cellular homeostasis. NO levels in Hippocampus were measured using the previous described method [24]. The basis of this method is the reaction of N-(1-naphthyl) ethylenediamine dihydrochloride and sulfonamide with the NO content of the samples and absorbance reading at 540 nm.

2.5.2. Ferric reducing antioxidant power (FRAP) assay

The antioxidant power of Hippocampus samples in studied groups was measured using the FRAP test. briefly, About 50 μL of brain extract and serum were added to premixed freshly prepared 1.5 mL of FRAP reagent (0.3 M, pH 3.6 acetate buffer: 20 mM $FeCl_3$: 10 mM TPTZ = 10:1:1). Then, the sample absorbance was recorded at 593 nm after 30 min incubation at 37 °C [25].

2.5.3. Lipid peroxidation (LPO) assay

LPO is the main indicator of the pattern of increased ROS in cellular damage. To measure the level of LPO in Hippocampus of studied groups, Malondialdehyde (MDA), was measured as the main product of LPO in the reaction with thiobarbituric acid at a wavelength of 532 nm [26].

2.6. Sexual hormones titer

The hypothalamic-pituitary-adrenal (HPA) axis has a significant effect in the pathophysiology of depressive disorder. On the other hand, the secretion of sex hormones is one of the important aspects of HPA axis activity. For this reason, Testosterone and estrogen level in serum of rats were measured using the ELISA kits (Diametra 20090 Segrate Milano Italy).

2.7. Real time PCR

To analyze the level of gene expression, total RNA was extracted from the Hippocampus of studied rats using TRIZOL reagent (Invitrogen Co) according to the manufacturer's instructions. One microgram of extracted RNA was used for cDNA synthesis using the Prime Script RT kit. Real Q Plus 2x Master Mix Green kit (Ampliqon Co.) was used for real time PCR test. Each samples were run in duplicates. The list of reverse and forward primers was mentioned in Table .1. Thermal alteration cycles consist of initial activation at 95 °C for 30 s; the following steps were extension/annealing for 45 cycles (5 s at 95 °C and 20 s at 60 °C). β-actin was considered as the reference gene and $2^{-\Delta\Delta ct}$ formula used to calculate related expression.

2.8. Histopathological assessment

After performing cervical dislocation of rats, the total extracted brain was fixed in 4% buffered formalin, and stored for further exams. The dehydration process was done using sequential dehydration using 50, 70, 80, 90% and absolute alcohol. After the clearing and paraffin fixation of the tissue, 8–10 μm slices were prepared and installed on slides. Hematoxylin and eosin staining (H&E) was performed. The histopathological alterations were studied under optical microscope [22].

2.9. Statistical analysis

The results are presented as the mean ± SD. Significant differences were set at the value of $p < 0.05$ using SPSS 17.0 statistical software (SPSS Inc., IL, USA) of two-way ANOVA analysis for independent samples were applied to test potential differences between social condition (control) and PWSI rats on all variables.

3. Results

3.1. The effects of sex difference on behavioral alteration

3.1.1. The effects of PWSI on locomotor activity in male and female rats

The results of two-way ANOVA analysis showed the effect of PWSI [$F(1, 38) = 0.00031$; $p > 0.05$] and gender [$F(1, 38) = 40.7$; $P < 0.001$] on horizontal activity. The result of OFT test showed that there is no significant difference between the studied groups in horizontal activity (Fig. 1a, $P > 0.05$). The results of two-way ANOVA analysis showed the effect of PWSI [$F(1, 38) = 36.66$; $p < 0.01$] and gender [$F(1, 38) = 11.48$; $P < 0.001$] on horizontal activity. Vertical activity in all PWSI groups located in the diestrus phase and male rats had a significant decrease compared to the control groups (Fig. 1b, $P < 0.01$).

3.1.2. The effects of PWSI on anxiety behavior in male and female rats

The results of two-way ANOVA analysis showed the effect of PWSI [$F(1, 38) = 12.22$; $p < 0.001$] and gender [$F(1, 38) = 49.56$; $P < 0.001$] on FST. The examination of anxiety behavior between groups showed that a significant difference between PWSI and control female rats in the estrous phase were not observed (Fig. 2. $P > 0.05$), while PWSI male and PWSI female rat in the diestrus phase compared to the control groups, spent a significant and longer time in the dark arm of the maze and had more anxiety (Fig. 2. $P < 0.01$).

3.1.3. The effects of PWSI on depressive symptoms in male and female rats

The results of two-way ANOVA analysis showed the effect of PWSI [$F(1, 38) = 13.25$; $p < 0.001$] and gender [$F(1, 38) = 3.62$; $P > 0.05$] on FST. Evaluating the effect of PWSI on depression using the FST test showed that there is no significant difference between PWSI female rats in the estrous phase and control. However, female rats in diestrus phase of PWSI group and male PWSI rats showed higher and significant levels of depression compared to the control groups (Fig. 3. $P < 0.001$).

3.1.4. The effects of PWSI on grooming behavior in male and female rats

The results of two-way ANOVA analysis showed the effect of PWSI [$F(1, 38) = 47.74$; $p < 0.001$] and gender [$F(1, 38) = 12.704$; P

Table 1
Primer sequences used for real-time PCR assay.

Name	Sequence (5′–▶3′)	Gene Bank accession number
nNOS	CTGACCTGTTGCTTAGGGATA TCATCTGCTCATTGCCATTCG	NM_039089061.1
iNOS	AGCCACGGATATTTAGAGTG CAGAGAAAGAGCACATAGAC	NM_039085203.1
β actin	CTAGGCACCAGGGTGTGATG GCACAGGGTGCTCCTCAG	NM_007393.5

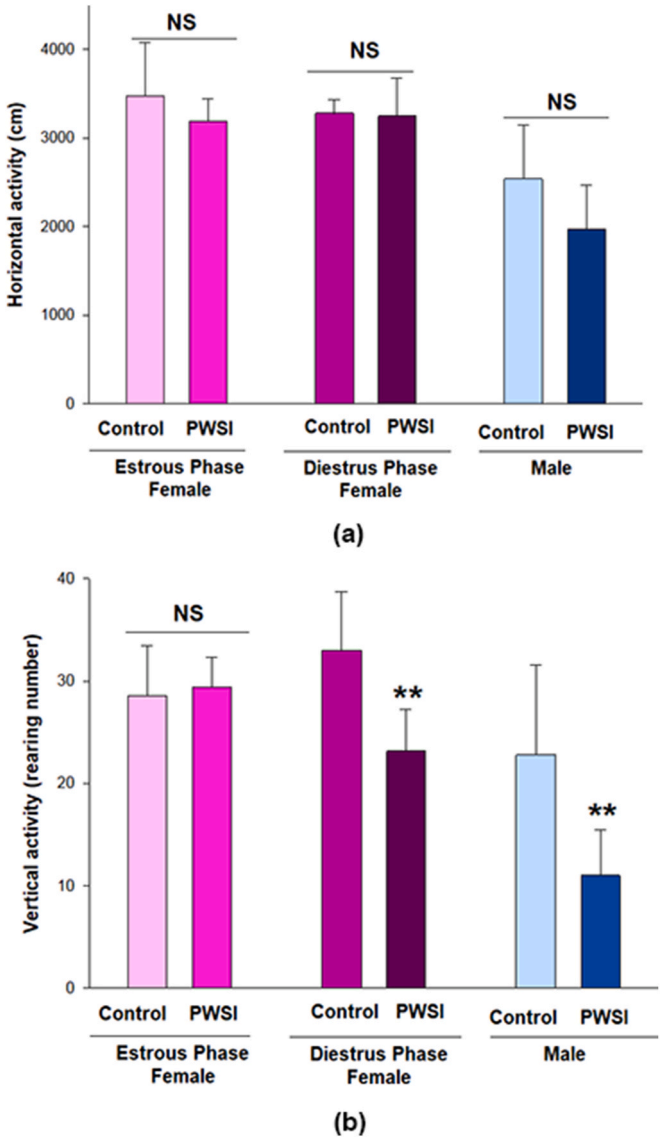

Fig. 1. The effects of PWSI on (a) Horizontal and (b) vertical activity in male and female rats. Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 7–8); NS = non-significant, **p < 0.01].

< 0.001] on grooming activity. As Fig. 4a depicts, the grooming behavior initiated significantly later in diestrus female and male PWSI rats when compared to the control group (P < 0.001). The same pattern was not repeated in in estrous female rodents (P > 0.05). Measuring the duration of grooming behavior of rats showed that the cleaning activity in all PWSI groups was significantly reduced compared to the control groups (Fig. 4b, P < 0.001).

3.1.5. The effects of PWSI on tendency to explore unfamiliar objects in male and female rats

The results of two-way ANOVA analysis showed the effect of PWSI [F(1, 38) = 14.382; p < 0.001] and gender [F(1, 38) = 0.007; P > 0.05] on NOR outputs. The results of the NOR test show a significant reduction in the discrimination index between diestrus female and male PWSI rats when compared to the control groups (Fig. 5; P < 0.05 and P < 0.001 respectively). However, the contrast between PWSI estrous female rats and their related control remained insignificant (Fig. 5; P > 0.05).

3.2. The effects of six difference of PWSI induced rats on biochemical parameters

3.2.1. The effects of PWSI on nitric oxide levels in male and female rats

The results of two-way ANOVA analysis showed the effect of PWSI [F(1, 14) = 1.166; p > 0.05] and gender [F(1, 14) = 5.328; P <

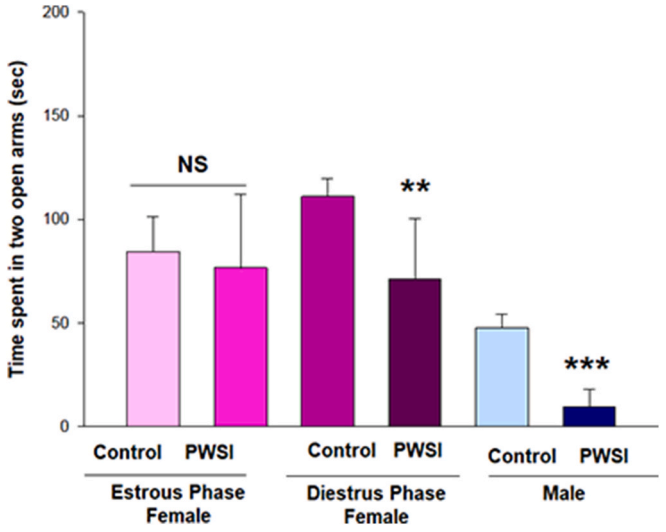

Fig. 2. The effects of PWSI on anxiolytic behavior in male and female rats. Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 7–8); NS = non-significant, **p < 0.01, ***p < 0.001].

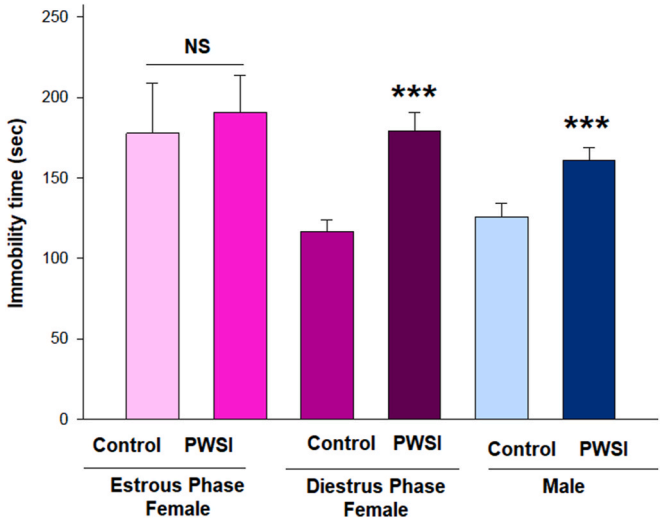

Fig. 3. The effects of PWSI on depressive symptoms in FST in male and female rats. Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 7–8); NS = non-significant, ***p < 0.001].

0.05] on NO level. PWSI induction had no effect on the level of NO in the Hippocampus of intervention rats of both sexes compared to the control group (Fig. 6; P > 0.05).

3.2.2. The effects of PWSI on FRAP levels in male and female rats

The results of two-way ANOVA analysis showed the effect of PWSI [F(1, 14) = 12.298; p < 0.01] and gender [F(1, 14) = 2.538; p > 0.05] on FRAP level. The Post hoc analysis showed significant decrease caused by PWSI in male and female rats in the diestrus phase compared to the control groups. No significant difference was observed between female rats in estrous phase (Fig. 7; P < 0.001 and P < 0.01, respectively).

3.2.3. The effects of PWSI on MDA levels in male and female rats

The results of two-way ANOVA analysis showed the effect of PWSI [F(1, 14) = 12.298; p < 0.01] and gender [F(1, 14) = 2.538; p > 0.05] on MDA level. The Post hoc analysis showed significant increase in female PWSI rats in the diestrus phase and male PWSI rats compared to the control groups (Fig. 8; P < 0.01 and P < 0.001, respectively). Also, the significant difference was not observed

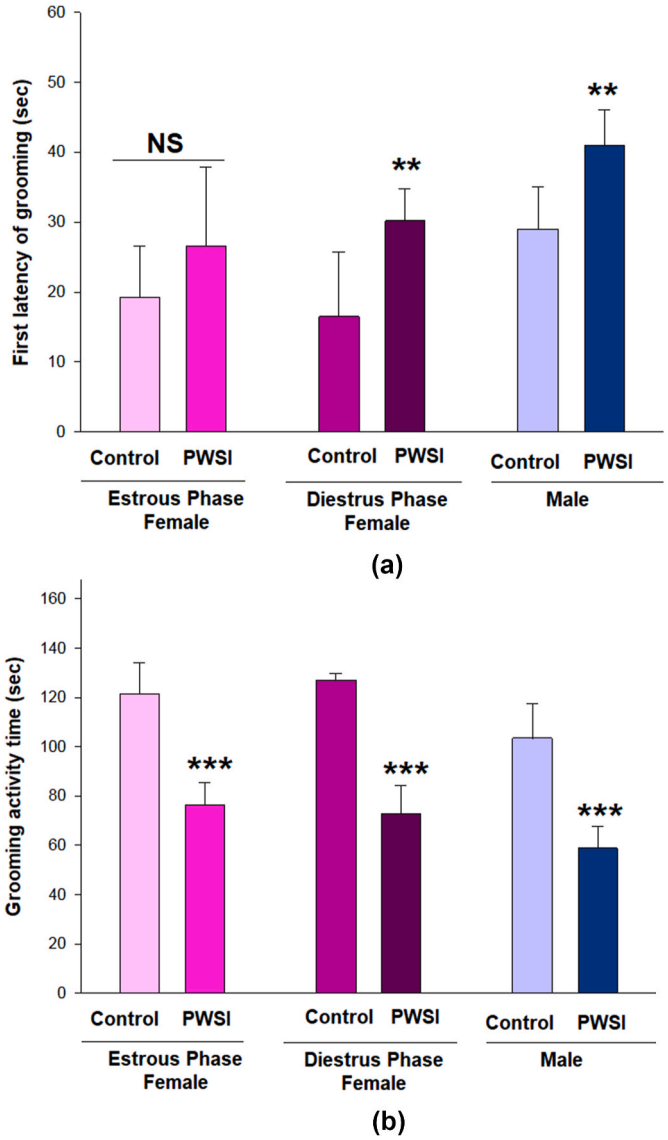

Fig. 4. The effects of PWSI on depressive symptoms in splash test in male and female rats. (a) First latency of grooming (b) Grooming activity time.Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 7–8); NS = non-significant, **p < 0.01, ***p < 0.001].

between female PWSI rats in estrous phase and social condition rats or control groups (Fig. 8; P > 0.05).

3.3. The effects of PWSI and gender on estradiol and testosterone levels in male and female rats

The results of two-way ANOVA analysis showed the effect of PWSI [F(1, 26) = 0.00169; p > 0.05] and gender [F(1, 26) = 41.719; p < 0.001] on estradiol level. As showed in Fig. 9a, the estradiol level did not go under any significant change in any of the three groups compared to the relative control. However, The two-way ANOVA analysis showed the effect of PWSI [F(1, 26) = 123.53; p > 0.05] and gender [F(1, 26) = 1813.42; p < 0.001] on testosterone level as in Fig. 9b, the level of testosterone has significantly dropped (P > 0.001 and P > 0.01, sequentially).

3.4. The effects of PWSI on gene expression of iNOS and nNOS in the brain of male and female rats

The effect of PWSI [F(1, 14) = 30.44; p < 0.001] and gender [F(1, 14) = 15.59; p < 0.001] on iNOS expression indicated a significant increase in the expression level of iNOS in PWSI groups compared to the control groups (Fig. 10a; P < 0.01). The two-way ANOVA analysis the effect of PWSI [F(1, 14) = 10.6; p < 0.01] and gender [F(1, 14) = 2.68; p > 0.05] on iNOS expression. Unlike

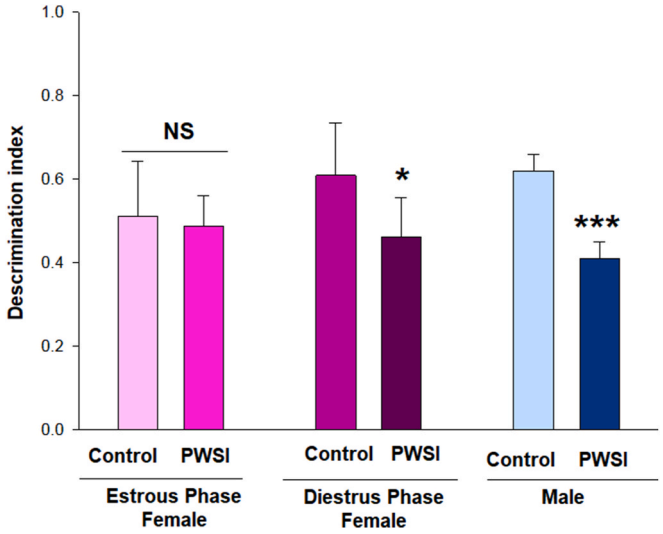

Fig. 5. The effects of PWSI on tendency to explore unfamiliar objects in NOR test in male and female rats. Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 7–8); NS = non-significant, *p < 0.05, ***p < 0.001].

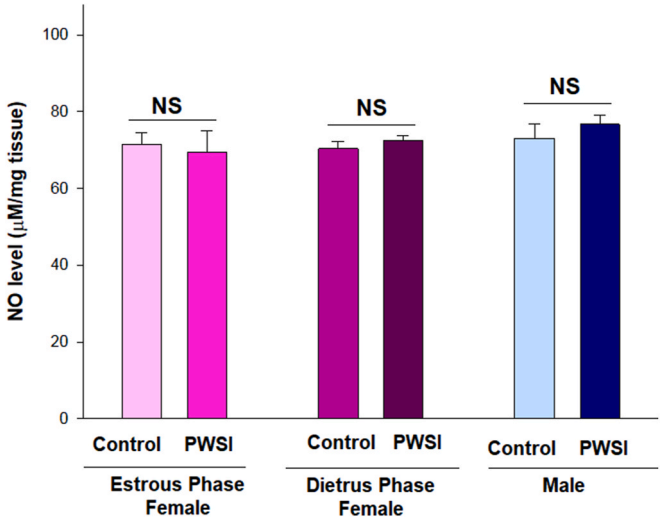

Fig. 6. The effects of PWSI on nitric oxide levels in brain of male and female rats. Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 3–4); NS = non-significant].

iNOS, the level of nNOS expression in female PWSI rats in diestrus phase and male PWSI rats was significantly decreased compared to the control groups (Fig. 10b; P < 0.001). There was no significant difference in the expression level of nNOS in female PWSI rats in estrous phase and control group.

3.5. The effects of six difference on histopathological data

As shown in Fig. 11, following PWSI induction, the basophilic necrosis, vacuolation and accumulation of inflammatory cells were observed only PWSI in diestrus phase of female rats and male rats in PWSI condition. However, female PWSI rats in estrous phase showed none of the aforementioned degeneration pattern which was presented in diestrus phase of female rats.

4. Discussion

This study observed the impact of social isolation on the level of neural antioxidants, oxidative stress and NO homeostasis. The relative fluctuation of sexual hormones in relation to gender can alter the pattern of cognitive and biochemical mitigation in PWSI rats

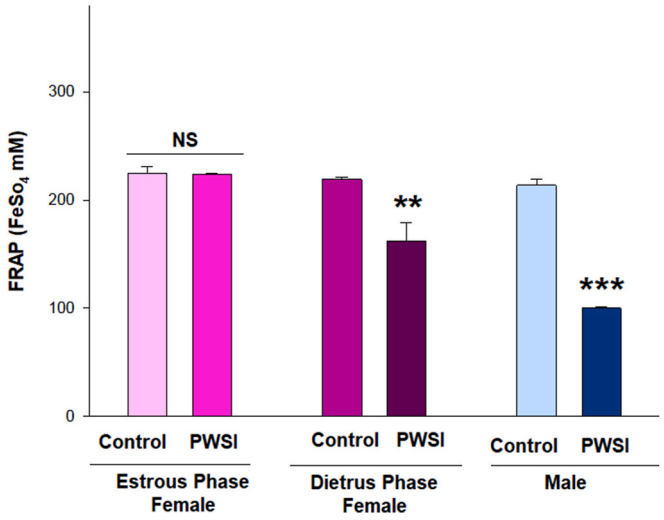

Fig. 7. The effects of PWSI on FRAP level in brain of male and female rats. Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 3–4); NS = non-significant, (**p < 0.01, ***p < 0.001].

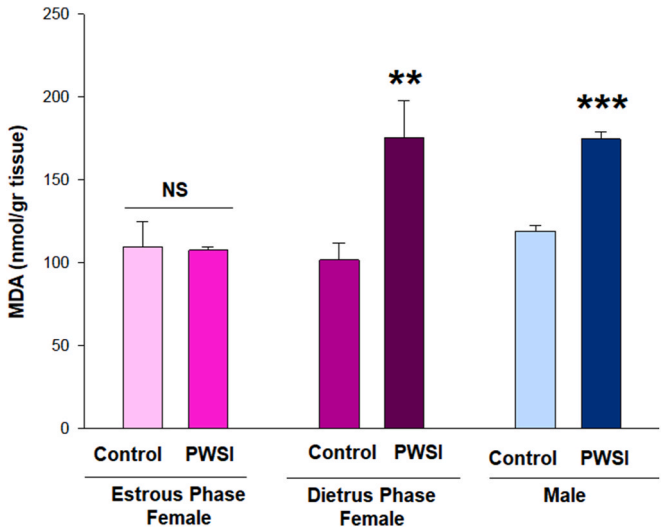

Fig. 8. The effects of PWSI on MDA amount in brain of male and female rats. Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 3–4); NS = non-significant, (**p < 0.01, ***p < 0.001].

with more potent impact on females. There is a direct correlation between social anxiety and loneliness with depression [27]. There is a direct correlation with ovarian hormones level in perimenopause phase, and the severity of depression signs [28]. In the present study, PWSI rats demonstrated lower vertical activity, which is indicating rodents' curiosity, and rodents had lesser presence in open limbs of T-maze as representation of higher anxiety rate. As the result, facing PWSI condition has caused rodents to become more isolated and suppress their exploring habit [29]. This case was only applied about PWSI rats in diestrus female and male, where low level of progesterone and estradiol in female and male might amplified the intensity of PWSI on behavioral factors [30].

The result of depression-like behavior coincided with social anxiety manifestation, later tests related to investigation of anhedonia and survival behavior revealed that PWSI could mitigate aforementioned factors by decreasing grooming activity and increasing immobility in splash test and FST, respectively. The latency of the first grooming and immobility time, however, did not alter in estrous phase, these results also correlated with the surge of estradiol and progesterone in estrous female rats. The estrous cycle in rodents is resemble to menstrual cycle in human consists of proestrus, estrus, metestrus and diestrus phase and lasts 4–5 days. The proestrus phase depicts the highest estradiol secretion, then the decline of estradiol cause ovulation and initiation of estrus phase. The metestrus and diestrus phases resemble the late human phase of menstruation, when progesterone content rises [31]. While additional

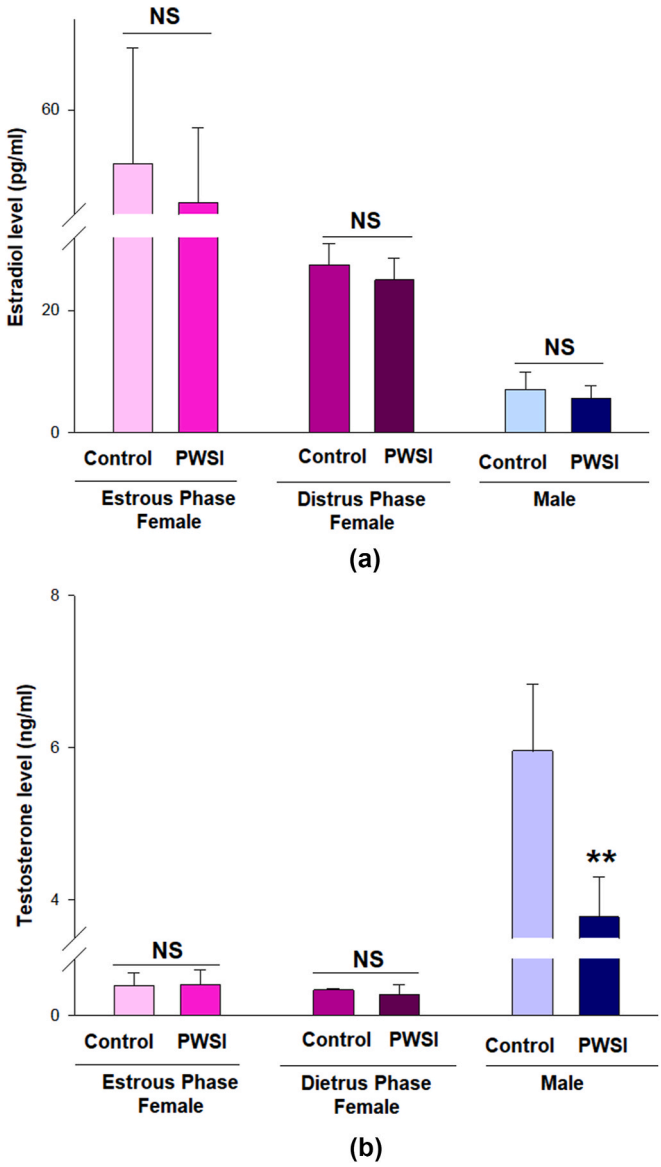

Fig. 9. The effects of PWSI on (a) estradiol and (b) testosterone levels in blood sample of male and female rats. Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 3–4); NS = non-significant, **p < 0.01].

application of progesterone demonstrated to resolve depressive-like behavior in diestrus female rats [32], the drop in estradiol level has direct influence on neural plasticity and BDNF factor then affecting left amygdala activity. This effect was more dominant in stress and isolation induced after early life stress [33,34]. The study showed the peak level of estradiol and progesterone may induce protective effects against social isolation behavioral imparity in female rats during estrous phase (estrous combined with proestrus), while the depressive-like effect in male rats did not show fluctuation (do [35]). The PWSI made rats showed significantly lower level of testosterone compared to control. The role of testosterone in neurogenesis in dentate gyrus of hippocampus, and application of exogenous testosterone did not recover the behavioral and neural generation factors [36].

Later on, we have evaluated NO in intracellular and extracellular level, while no alteration was noticed in intracellular level. However, significant drop in extracellular NO presented itself in male rats' brain with significant higher expression level for eNOS and iNOS, this effect led to lower quantity of NO presented itself in plasma fluid under PWSI induction. In related studies, expression of nNOS has decreased in long exposure to social isolation and crowding stress in hypothalamus which known to correlate with HPA-axis performance [37]. These effect were also coinciding with systemic surge of IL-6, IL-1β and TNF-α as markers for inflammation [38].

In case of estrous cycle, NO level has not made any significant alteration nether in estrous nor diestrus phase. In contrary, the expression level of iNOS in both phases, and the expression of eNOS and nNOS had major fluctuation during diestrus phase in PWSI

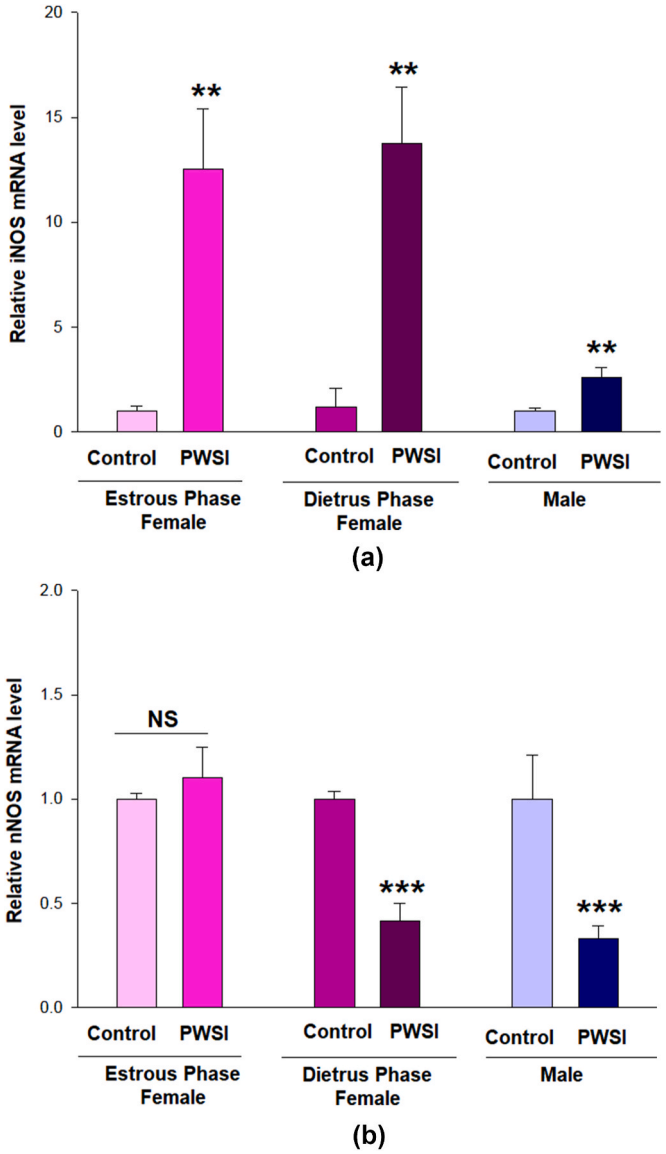

Fig. 10. The effects of PWSI on gene expression of (a) iNOS and (b) nNOS in the brain of male and female rats. Values are expressed as the mean ± SD and were analyzed using student's t-tests for independent samples. Significant difference compared to their control groups [(n = 3–4); NS = non-significant, **p < 0.01].

group (while former raised the latter fall). Later studies demonstrated the difference between the two sex in NO level and neurotrophins expression to be more impacted in male than female rats [39]. However, our study reveals that estrous cycle is the main factor in severity of social isolation's behavioral and biochemical alteration in female rats. These alteration seems to effect ovarian function as to alternate 17-beta estradiol and progesterone level during proestrus phase [40].

In conclusion, social isolation may induce major alteration in NO homeostasis; oxidative stress and deprivation of cellular antioxidants these effect induce alterations on sexual hormones in both genders of rodents. The consequent fluctuation of estradiol and progesterone alters the severity of social isolation in female rats.

5. Study limitations

For further investigations it is recommended to observe the role of gender in alternation of immune system performance in rodents under influence of PWSI, the immune system plays a significant role in pathophysiology of depressive disorder. The neuroplasticity has a major role in formation of intracellular connection and formation of memory, the markers of neural plasticity undergo significant drop as a result of PWSI induction, it is recommended to study these factors in both genders in further studies. Though rodent has been utilized in various studies to mimic biochemical and behavioral patterns of human diseases, it is highly recommended to convey

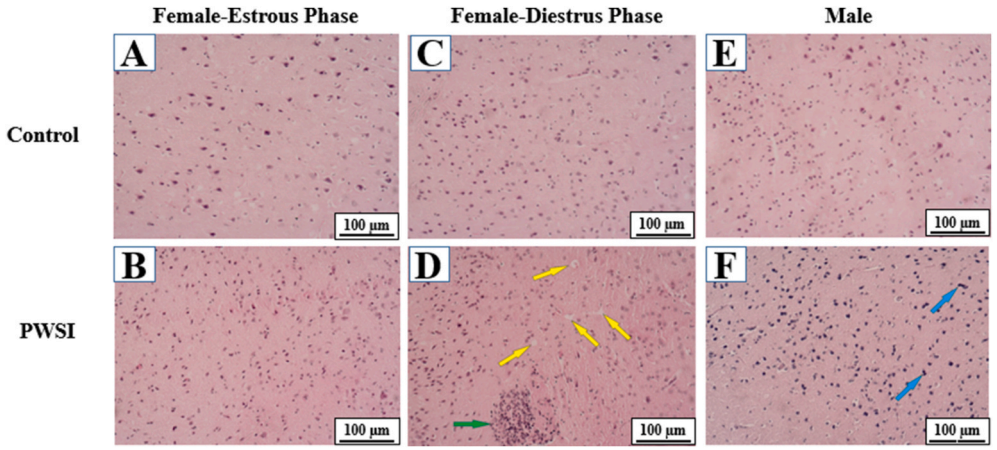

Fig. 11. Effects of various housing conditions and sex difference on hippocampal histopathology. H&E staining, × 400. Grading of histopathological changes (Scoring) was done as follows: NO (0); Mild (1); Moderate (2); and Severe (3). Microglial nodule (blue arrows), basophilic necrotic neuron (yellow arrows) and vacuolization (red arrows) was observed. A- Estrous Phase of female control rats: histological structure was normal (Grade 0) B- Estrous Phase of female PWSI rats: histological structure was normal (Grade 0) C- Diestrus Phase of female control rats: histological structure was normal (Grade 0) D- Diestrus Phase of female PWSI rats: vacuolation (yellow arrows) and accumulation of inflammatory cells (green arrow) were observed in grade 2. E– Male control rats in social condition: histological structure was normal (Grade 0) F- Male PWSI rats: Basophilic necrosis (blue arrows) were observed in grade 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

relative study on social isolated patients.

Funding

the deputy of research of Zanjan University of Medical Sciences supported this work (Grant NO: A-12-769-46& 47).

Ethics approval and consent to participate

All procedures were carried out following the regulations of the University and the Guide for the Care and Use of Laboratory Animals of National Institutes of Health (Ethical Code: ZUMS.REC.1399.178) and Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press). Full efforts was made to reduce animals' use and advance their welfare.

Consent for publication

All authors are agreed to publish this manuscript.

Data availability statements

The data that support the findings of this study are available on request from the corresponding author, [Mir-Jamal Hosseini]. The data are not publicly available due to our critical plan to investigate different studies based on our design.

CRediT authorship contribution statement

Soroush Bijani: Writing – review & editing, Writing – original draft, Software, Investigation, Data curation. **Fatemeh Sadat Kashfi:** Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Investigation, Formal analysis, Data curation. **Sadaf Zahedi-Vanjani:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Keivan Nedaei:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Data curation. **Ali Sharafi:** Writing – original draft, Supervision, Software, Project administration, Methodology, Investigation, Data curation. **Ali Kalantari-Hesari:** Writing – original draft, Visualization, Supervision, Methodology, Investigation, Data curation. **Mir-Jamal Hosseini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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