Research in Molecular Medicine

# Responsiveness to the Effect of Fluoxetine in Male and Female Rats Exposed to Single Prolonged Stress: A Behavioral, Biochemical, Molecular and Histological Study

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## ABSTRACT

**Background:** Selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine are the first-line choice in patients with post-traumatic stress disorder (PTSD). Animal studies have indicated that chronic fluoxetine exposure leads to persistent behavioral changes and neuroplasticity in the hippocampal formation and cortex. Previous studies revealed that adult female rats respond differently to trauma from adult males. Here, we have raised the question of whether a difference is observed in the response of both sexes to the fluoxetine treatment.

**Materials and Methods:** In a rat model of PTSD, the single prolonged stress (SPS) model, rats were exposed to SPS (restrained for 2 h, forced to swim for 20 minutes, and exposed to ether anesthesia) and then were kept undisturbed for 14 days. After that, SPS rats were subjected to chronic treatment with fluoxetine (10 mg/kg -28 days), followed by behavioral (object location memory test [OLMT] and object recognition memory test [ORMT]), and biochemical tests, in which (serum insulin-like growth factor 1 [IGF-1] levels were measured using a Rat ELISA Kit), and the messenger ribonucleic acid (mRNA) expression of anti-apoptotic factor (B-cell lymphoma 2 [Bcl-2]) and pro-apoptotic makers (Bax, and caspase 3) were determined by using reverse transcription-polymerase chain reaction (RT-PCR) method and histological assessments by Golgi-Cox staining.

**Results:** Male and female rats with PTSD, show a reduction of the levels of serum IGF-1, impaired spatial memory in a recognition location memory task and enhanced apoptotic-related factors expression in the hippocampus, and decreased hippocampal dendritic branches. Fluoxetine treatment alleviated these abnormalities in male and female SPS rats, but fluoxetine had no sex-dependent effects on these factors.

**Conclusion:** Our findings support that fluoxetine treatment can prevent the harmful effects of traumatic events in an animal model of PTSD in both sexes.

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## Introduction

Post-traumatic stress disorder (PTSD) is a chronic psychiatric disease with high prevalence and morbidity [1, 2]. This disease occurs when a person experiences traumatic events and is associated with characteristic neurobiological changes [3, 4]. PTSD can be characterized by revisiting the first trauma and is accompanied by some signs, such as anxiety, insomnia, nightmares, memory loss, behavioral changes, hypervigilance and hyper-arousal, crowd or social avoidance, cognitive deficits, susceptibility to infections, immune suppression, autoimmune diseases, depression, respiratory disease, hypertension, and chronic pain [4, 5].

When people experience a traumatic event during their lifetime, they show an acute response to the trauma, but only some of these people ultimately develop PTSD, and others fully recover after the first acute response [6]. Patients with PTSD have shown changes in the volume and density of gray matter in the cortex, hippocampus, corpus callosum, and prefrontal cortex; it plays a key role in their behavior and emotions [7]. Dysfunction of the prefrontal cortex -hippocampus-amygdala circuitry is associated with impaired learning and memory in this disease [8, 9]. Although animal studies have indicated that impaired neuronal plasticity and enhanced apoptosis may contribute to the molecular mechanisms of the PTSD pathological process, it is necessary to identify the main mechanisms involved in this disease. It has been shown that these behavioral changes likely occurred via control systems, such as regulation of the monoamine system, such as the 5-hydroxytryptamine (5-HT) system [10].

Two common treatments exist for PTSD, psychotherapy and pharmacotherapy, and a combination of these. Selective serotonin reuptake inhibitors (SSRI) [11] are recommended as the first-line medications in most treatment guidelines [12]. Desipramine, fluoxetine, paroxetine, phenelzine, risperidone, sertraline, and venlafaxine are members of this group [13] and seem to be a good suggestion for patients with PTSD. Besides SSRIs drugs, some other drugs, such as benzodiazepines, atypical antipsychotics, and antidepressants have shown a good impact on the clinical treatment of patients [14]. Serotonin, or 5-hydroxytryptamine (5-HT) is a neurotransmitter with some essential functions, including reward, memory, and numerous physiological processes, such as vomiting and vasoconstriction, modulating mood, cognition, and learning. In PTSD disorder, the level of platelet serotonin decreases, as well as the function of single nucleotide polymorphism (SNPs) in the serotonin transporter gene and the serotonin 2A receptor gene changes [15]. Serotonin has a crucial role during embryogenesis and the differentiation of stem cells into neuron cells [16]. In patients with PTSD, the regulation of function and production of serotonin are impaired [17].

Insulin-like growth factor 1 (IGF-1) is a polypeptide composed of 70 amino acids and has two chains, a and b connected by a disulfide bond. It is produced by different body areas, which justifies its numerous effects. For example, the liver frequently emits it and plays the role of endocrine part. Furthermore, it is produced in the cartilage but has paracrine effects [18]. Growth, development, metabolism, and longevity regulation are affected by IGF-1 [19]. Local secretion of this factor occurs in the cortex of the brain, hippocampus, cerebellum, and hypothalamus [20].

It has been demonstrated that fluoxetine has different neurological and cognitive effects on the nervous system [21] and can increase the proliferation of neurons that promote neurogenesis in the central nervous system (CNS) [22]. Sex contributes to susceptibility to stress-related disorders. The prevalence of anxiety and stress-related mental disorders (PTSD, depression, anxiety disorders, and social anxiety or phobia) in women is nearly twice that of men [23, 24]. Studies conducted on gender differences in response to stress have shown differences in the neural circuits effective in emotional reactions. Epidemiological studies have suggested that women may have a higher risk for anxiety disorders and their symptoms may worsen during different phases of reproductive life, such as puberty, menstruation, postpartum, and menopause [25]. Gender differences are observed in the effects of antidepressants and clinical manifestations of psychiatric disorders. Sex hormones in females can affect pharmacokinetics and the efficacy of antidepressant drugs [26] and women in reproductive years respond better to fluoxetine than men [27]. Our previous study on patients with PTSD showed the positive effect of fluoxetine on reducing pro-apoptotic factors in male single prolonged stress (SPS) rats [28]. Given that PTSD leads to damage to the hippocampus and changes in its normal function, one of the therapeutic goals in the present study in such a disease is to improve trauma-related injuries, such as spatial and cognitive memory and cell apoptosis, and alter the secretion of IGF-1c as a neurotrophic factor in two genders. It is still not clear whether the response to treatment is different in both genders.

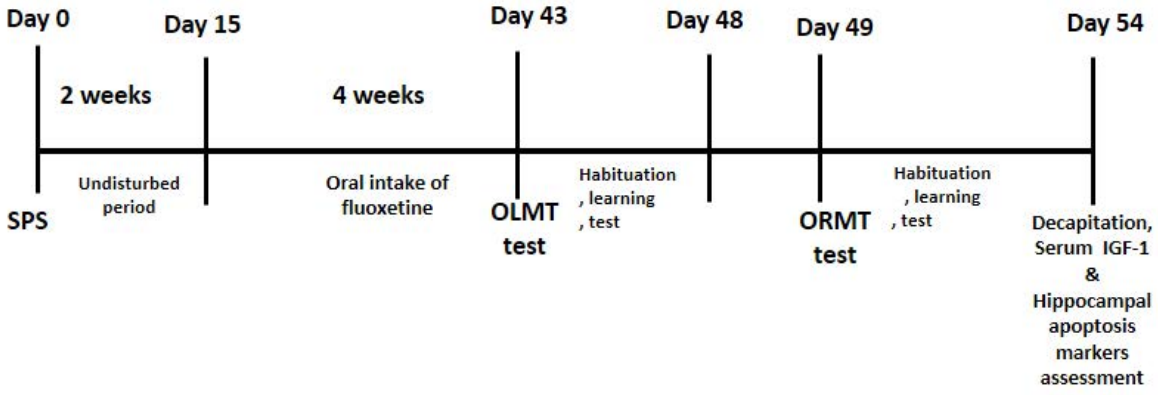



**Figure 1.** Timelines of experiments

## Materials and Methods

### Animals

Eighty male and female Wistar rats with an average weight of 200-250 g were obtained from the animal lab research center (Mazandaran University of Medical Sciences, Sari, Iran). Male and female rats (n=80) were divided into the following 8 groups, (n=10 per group):

1- Male/Sham-Vehicle (VEH)

2- Male/Sham-Fluoxetine (FLX)

3- Male/SPS-Vehicle (VEH)

4- Male/SPS-Fluoxetine (FLX)

5- Female/Sham-Vehicle (VEH)

6- Female/Sham-Fluoxetine (FLX)

7- Female/SPS-Vehicle (VEH)

8- Female/SPS-Fluoxetine (FLX).

The number of rats in each group was determined based on the pilot study and the other studies [28, 29]. The rats were housed in groups of five within Macrolon transparent polycarbonate cages (61.28×43.5×21.6 cm) with a wire top.

Male and female rats were kept in separate rooms. They were kept in a room on a 12-h light/dark schedule (lights on at 6:00 AM) and constant temperature (24±2°C), 60% humidity with food and tap water ad libitum.

### Drug

Fluoxetine hydrochloride (Dr. Abidi Company, Tehran City, Iran) was dissolved in the water before use in a dose of 10 mg/kg/day for 28 days. The control groups received water alone and solutions were refreshed daily. The drug dose was chosen based on previous studies [28, 30]. Before drug administration, water consumption was measured for every cage from the water bottle and converted to a daily dose per mg/kg body weight. All drug-receiving groups were weighed on the first day of every week. Oral intake of fluoxetine was the best administration method because this mimics its clinical use and prevents the stress associated with injections or gavage.

After the period of drug intervention, and conducting behavioral tests, the five rats from each group of animals were deep anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) and decapitated, by cutting the jugular vein, trunk blood was collected in tubes and centrifuged (4000×g for 5 minutes) and the serum was stored at -80°C until used for the IGF-1 assay. Serum IGF-1 levels were assessed using Rat IGF-1 ELISA kits (ZellBio, Germany) according to the manufacturer's recommendations. The assay range is 30–960 ng/mL. The sensitivity of the assay was 1.5 ng/mL.

The brains of these 5 animals were placed in Golgi-Cox solution for tissue study. In the other 5 rats, the hippocampi were removed and then immediately frozen at -80°C to prepare homogenates with a homogenizer. The hippocampus was cut into small pieces and homogenized in phosphate-buffered saline, 7.4 pH by the homogenizer. The homogenate was centrifuged at 13000×g for 15 minutes at 4°C. Thereafter, supernatants were aliquoted and stored at -80°C for the reverse transcription-polymerase chain reaction (RT-PCR) process (Figure 1).

## PTSD model

PTSD is caused by SPS. It was performed in three steps. The first step is a restraint for 2 hours. Then they are forced to swim for 20 minutes in a clear acrylic cylinder (240 mm in diameter and 500 mm in height), two-thirds of which is filled with water. After the forced swimming, it takes 15 minutes for the animals to recover. In the last step, they lose consciousness with diethyl ether and remain undisturbed in their cages for 14 days. Fourteen days after SPS, to study the effects of fluoxetine, all animals in the SPS and Sham groups will be divided into groups receiving and not receiving the drug [31, 32].

## Memory assessment tests

### Recognition location memory test

An object location memory test (OLMT) has been developed to test spatial memory in rats [33]. We used two small dolls (5 cm diameter and 10 cm height) in a black open field (71×71×50 cm) under dim light (70 lux), the distance from the walls was 11 cm. The open field and the objects were between trials using 10% ethanol.

This test has three phases. In the first phase (habituation), rats were habituated to the experimental apparatus and allowed to explore it for 10 minutes every day for 3 days without objects, in the second phase (learning), two objects were placed in the open field, and the animals are exposed to objects for 5 minutes. In the third phase (test), which was conducted one day later, one object was moved to a new location and the time spent exploring the objects at the familiar and new locations was recorded for 5 minutes by a digital camera placed above the arena (for OLMT), and for object recognition memory test (ORMT), one day later. In the test phase, one novel object was moved to the same location and the time spent discriminating between the novel object (new object) and familiar object (the object that he was familiar with in the learning phase) was recorded for 5 minutes by a digital camera placed above the arena (Figure 1).

Time exploring of the displaced object reflects spatial memory and discrimination of the new object reflects recognition memory. Then, according to the following formula (Equation 1), it will be calculated that if the differentiation index during the test is more than 50%, it indicates that it has spent more time in the new situation (or near the novel object).

1. (TN+TF/TN)

Where, TN: Time exploring novel location (or novel object) and TF: Time exploring familiar location (or familiar object)

## Real-time PCR (reverse transcription-quantitative polymerase chain reaction [RT-qPCR])

RT-PCR test for pro-apoptotic (Bax, and caspase 3) and anti-apoptotic (Bcl2) proteins.

Homogenates were used to measure apoptosis factors. The first ribonucleic acids (RNAs) were extracted according to the manufacturer's instruction (DENA ZIST ASIA, Mashhad. Iran). The ribonucleic acids (RNAs) were used for complementary DNA (cDNA) synthesis with a commercial reverse transcription kit (Bioneer Inc., Seoul, South Korea). Quantitative real-time PCR was performed using the BIO-RAD IQ5 multicolor real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). CDNAs were stored at -20°C until the next use. Amplifications were performed in volumes of 20 µL of reaction mixture consisting of 0.4 µL of each primer (Bax, Bcl2, caspase 3, and β-actin as an internal control), 10 µL of 1X SYBR Green PCR master mix (Bioneer Inc., Seoul, South Korea) and 2 µL of cDNA. Table 1 presents primer sequences used in quantitative RT-PCR. Primers were designed using the Allele ID® version 7.5 (Premier Bio software, USA) and synthesized by Bioneer, Inc. (Seoul, South Korea). The initial denaturation was done at 95°C for 2 minutes followed by 45 cycles at 94°C for 30 s. The annealing temperature was 58°C for Bax, 59°C for Bcl-2, and 60°C for caspase 3 for 30 s. The final extension was performed at 72°C for 45 s. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All tests were performed in duplicate for each sample.

## Golgi–Cox staining protocol

Golgi staining remains a key method for studying neuronal morphology, being considered the most reliable method for demonstrating dendritic arborization [34].

### Preparation of solution

The impregnation stock solutions are prepared by dissolving 15 g from of the following chemicals in 300 mL dd-$H_2O$ (5%w/v) each:

1. Potassium dichromate

2. Mercuric chloride

3. Potassium chromate

**Table 1.** Primer sequences used in quantitative RT-PCR

| Gene | Primer Sequence | Product Size (Open Pair) |
|---|---|---|
| *Bax* | For. 5´-GGCTGGACACTGGACTTC -3´<br>Rev. 5´-CAGATGGTGAGTGAGGCA -3´ | 152 |
| *Bcl-2* | For. 5´-GTGGACAACATCGCTCTG-3´<br>Rev. 5´-AGACAGCCAGGAGAAATCA-3´ | 141 |
| *Caspase* | For. 5´-GACAACAACGAAACCTCC-3´<br>Rev. 5´-AGGGTAATCCATTTTGTAACTG-3´ | 122 |
| *ß-Actin* | For. 5´-GCCTTCCTTCCTGGGTAT-3´<br>Rev. 5´-GATCTTGATCTTCATGGTGCTA-3´ | 199 |

These solutions, stored in bottles at room temperature in the dark, are for long-term usage to prepare Golgi-Cox solution. Preparation of Golgi-Cox solution using the above compounds:

1. Fifty mL of the potassium dichromate solution is mixed with 50 mL of the mercuric chloride solution. A total of 2. 40 mL of the potassium chromate solution is added.

2. One hundred mL of dd-$H_2O$ is added. After mixing the solution, the bottle needs to be covered with aluminum foil and kept settling at room temperature for at least 48 h before use to allow precipitate formation. This solution can be used for up to 1 month.

### Statistical analysis:

Behavioral and biochemical data are expressed as the Mean±SE. The data were analyzed using a three-way analysis of variance (ANOVA) using SPSS software, version 21, followed by Tukey's post hoc test for multiple comparisons. Statistical differences were considered significant when $P<0.05$.

## 3. Results

### Object recognition memory test (ORMT)

Figure 2A shows data on the discrimination index (DI) in ORMT.

A three-way ANOVA (SPS×FLX×SEX) on the discrimination index demonstrated a significant effect of SPS ($F_{(1, 72)}=13.622$, P=0.0001), a significant effect of FLX ($F_{(1, 72)}=18.485$, P=0.0001), and no significant effect of SEX ($F_{(1, 72)}=1.471$, P=0.229).

Significant interaction between SPS×SEX ($F_{(1, 72)}=13.357$, P=0.0001) and SPS×FLX ($F_{(1, 72)}=18.363$, P=P=0.0001) and no significant interaction between FLX×SEX ($F_{(1, 72)}=0.121$, P=0.729) and FLX×SEX×SPS ($F_{(1, 72)}=1.860$, P=0.177).

Moreover, between-group comparisons indicated that the DI in the male/SPS-VEH group was significantly lower than in the male/Sham-VEH (P=0.0001), and fluoxetine significantly increased DI in male and female/SPS-FLX groups compared to SPS-VEH respectively (P=0.0001, P=0.021). A significant difference was observed between male/SPS-VEH and female/SPS-VEH (P=0.032). No significant difference was observed between female /SPS-VEH and female/sham-VEH.

### Object location memory test (OLMT)

Figure 2B shows data on the DI in OLMT.

A three-way ANOVA (SPS×FLX×SEX) on the data on the DI demonstrated a significant effect of SEX ($F_{(1, 72)}=12.763$, P=0.001), a significant effect of FLX ($F_{(1, 72)}=6.882$, P=0.011), and no significant effect of SPS ($F_{(1, 72)}=2.588$, P=0.112).

And no significant interaction was observed between SPS×SEX ($F_{(1, 72)}=0.150$, P=0.700) and SPS×FLX ($F_{(1, 72)}=1.141$, P=0.289) and FLX×SEX ($F_{(1, 72)}=0.017$, P=0.897) and FLX×SEX×SPS ($F_{(1, 72)}=0.000$, P=0.984).

Serum insulin-like growth factor 1 (IGF-1) assessment: Figure 3 shows data on serum IGF-1. A three-way ANO-

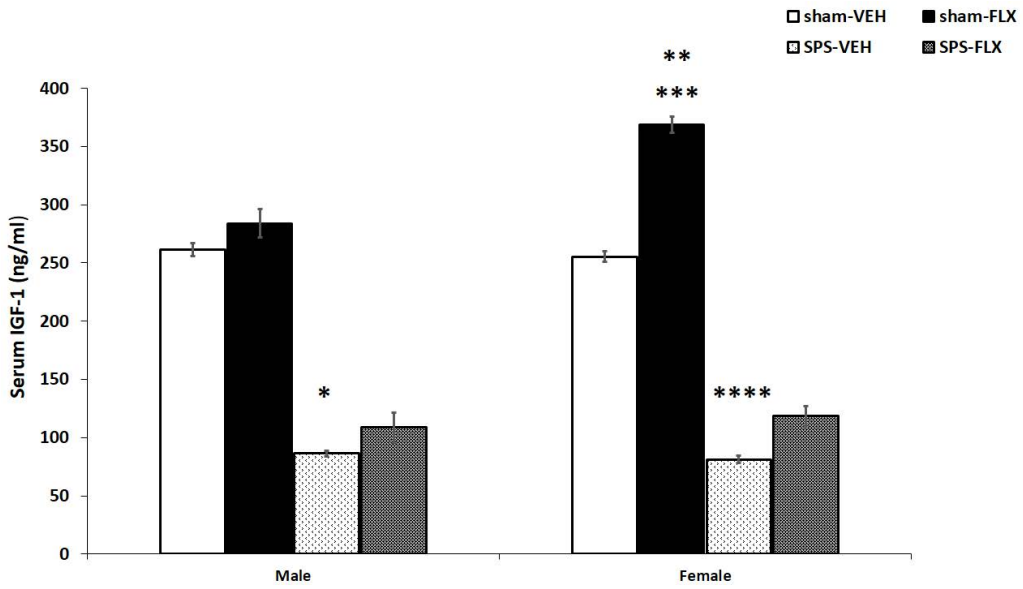



**Figure 2.** Serum IGF-1 levels in SPS rats subjected to fluoxetine, treatment.

* Male/SPS –VEH than Male/sham- VEH (P = 0.0001)

** Female/Sham –VEH than Female /sham- FLX (P = 0.0001)

*** Female/SPS –VEH than Female/Sham –VEH (P = 0.0001)

**** Female/Sham –VEH than Male/Sham –VEH (P = 0.0001)

VA (SPS×FLX×SEX) on the serum IGF-1 demonstrated a significant effect of SPS ($F_{(1, 72)}$=1208.9, P=0.0001), a significant effect of FLX ($F_{(1, 72)}$=77.093, P=0.0001), and a significant effect of SEX ($F_{(1, 72)}$=14.206, P=0.0001). A significant interaction was observed between SPS×SEX ($F_{(1, 72)}$=11.025, P=0.001) and SPS×FLX ($F_{(1, 72)}$=11.727, P=0.001) and between FLX×Sex ($F_{(1, 72)}$=22.622, P=0.0001) and FLX×SEX×SPS ($F_{(1, 72)}$=11.629, P=0.001).

Moreover, between-group comparison indicated that the serum IGF-1 significantly decreased in the SPS-VEH groups in male and female groups compared to Sham-VEH (P=0.0001).

FLX increased the serum IGF-1 significantly in female/Sham-FLX compared to female/Sham-VEH (P=0.0001). A significant difference was observed between male/Sham- FLX compared to female/Sham-FLX (P=0.0001).

Although fluoxetine increased the serum IGF-1 in SPS groups in male and female rats, it was not significant.

## Expression of messenger ribonucleic acid (mRNA) of apoptosis-related factors

### mRNA expression of bax

Figure 4A shows data on the mRNA expression BAX. A three-way ANOVA (SPS×FLX×SEX) showed a significant effect of SPS ($F_{(1, 32)}$=10.601, P=0.003), FLX ($F_{(1, 32)}$=43.708, P=0.0001) and SEX ($F_{(1, 32)}$=8.530, P=0.006) on the expression of BAX.

Also, a significant interaction was observed between SPS×FLX ($F_{(1, 32)}$=12.770, P=0.001), SPS×SEX ($F_{(1, 32)}$=6.637, P=0.015), FLX×SEX ($F_{(1, 32)}$=22.125, P=0.0001) and SPS×FLX×SEX ($F_{(1, 32)}$=4.874, P=0.035).

Moreover, between-group comparisons indicated that the expression of BAX in the male/SPS-VEH group was significantly higher than in the male/Sham-VEH (P=0.0001), and fluoxetine decreased the expression of BAX significantly in male/SPS-FLX groups compared to male SPS-VEH (P=0.0001). A significant difference was observed between male/SPS-VEH and female/SPS-VEH (P=0.0001).

### m-RNA expression of Bcl2

Figure 4B shows data on the expression of Bcl2. A three-way ANOVA (SPS×FLX×SEX) showed no significant effect of SPS ($F_{(1, 32)}$=0.228, P=0.637), and a sig-

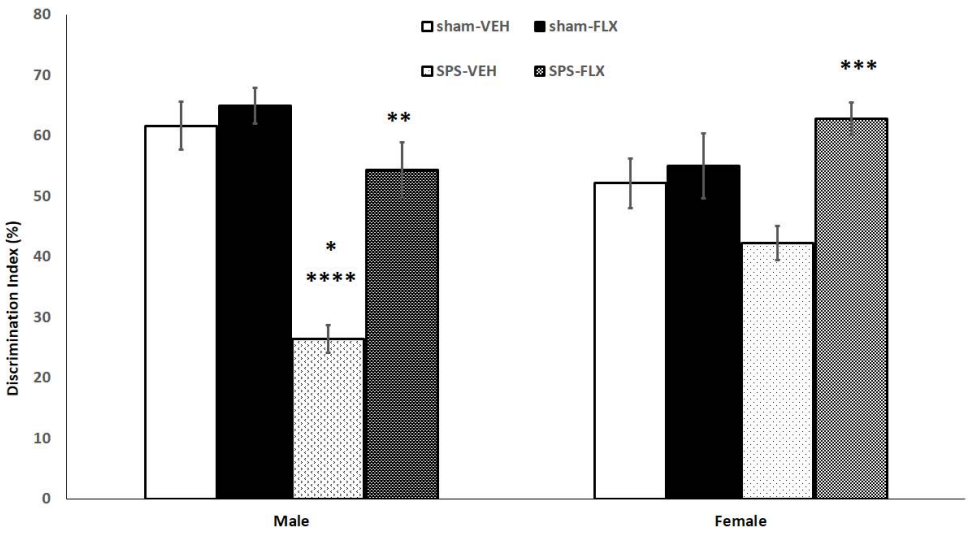



**Figure 3.** (A) Discrimination index

*Male/SPS –VEH than Male/sham- VEH (P = 0.0001)

**Male/SPS –FLX than Male/SPS –VEH (P = 0.0001)

***Female/ SPS –FLX than Female/SPS –VEH (P = 0.0001)

****Male/SPS –VEH than Female/SPS –VEH (P = 0.032)

nificant effect of FLX ($F_{(1, 32)}$=4.978, P=0.033) and SEX ($F_{(1, 32)}$=0.079, P=0.006) on the expression of Bcl2. Also, no significant interaction was observed between SPS×FLX ($F_{(1, 32)}$=2.841, P=0.102), SPS×SEX ($F_{(1, 32)}$=0.317, P=0.577), FLX×SEX ($F_{(1, 32)}$=2.837, P=0.102), and SPS×FLX×SEX ($F_{(1, 32)}$=3.806, P=0.060). Moreover, between-group comparisons indicated that fluoxetine significantly increased the expression of Bcl2 in female/SPS-FLX groups compared to female/SPS-VEH (P=0.013).

## Bax/Bcl2

Figure 4C shows data on the Bax/Bcl-2 ratio. A three-way ANOVA (SPS×FLX×SEX) showed a significant effect of SPS ($F_{(1, 32)}$=90.248, P=0.000), FLX ($F_{(1, 32)}$=204.927, P=0.000) and SEX ($F_{(1, 32)}$=5.109, P=0.031) on the expression of Bax/Bcl-2 ratio.

Also, a significant interaction was observed between SPS×FLX ($F_{(1, 32)}$=57.032, P=0.000), and no significant interaction was observed between SPS×SEX ($F_{(1, 32)}$=

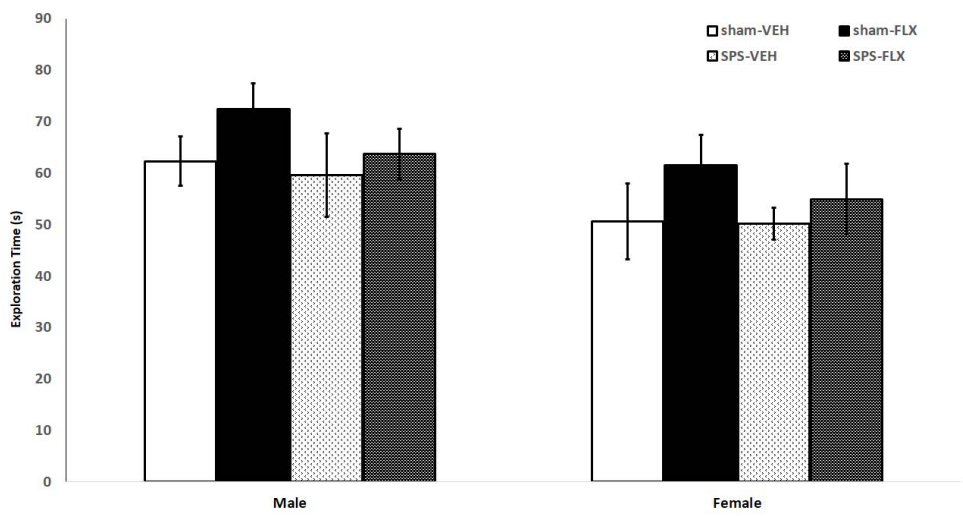



**Figure 3.** (B) Exploration Time

A, B. Recognition and Location memory task in SPS rats subjected to fluoxetine treatment

2.474, P=0.126), FLX×SEX ($F_{(1, 32)}$=0.177, P=0.676) and SPS×FLX×SEX ($F_{(1, 32)}$=1.046, P=0.314).

Moreover, between-group comparisons indicated that the Bax/Bcl-2 ratio in the male and female/SPS-VEH group was significantly higher than in the male and female/Sham-VEH groups (P=0.0001) and fluoxetine significantly decreased the Bax/Bcl-2 ratio in male and female/SPS-FLX groups compared to male and female/SPS-VEH groups (P=0.0001).

And also fluoxetine significantly decreased the Bax/Bcl-2 ratio in male/Sham-FLX groups compared to male/Sham-VEH groups (P=0.006).

### Caspase 3

Figure 4D shows data on the mRNA expression caspase 3. A three-way ANOVA (SPS×FLX×SEX) showed a significant effect of SPS ($F_{(1, 32)}$=65.650, P=0.0001), FLX ($F_{(1, 32)}$=180.653, P=0.0001), and no significant effect of SEX ($F_{(1, 32)}$=0.141, P=0.710) on the expression of caspase 3.

Also, a significant interaction was observed between SPS×FLX ($F_{(1,32)}$=100.465, P=0.0001), and no significant interaction between SPS×SEX ($F_{(1, 32)}$=0.004, P=0.949) and between FLX×SEX ($F_{(1, 32)}$=2.921, P=0.097) and SPS×FLX×SEX ($F_{(1, 32)}$=1.507, P=0.229).

Moreover, between-group comparisons indicated that the expression of caspase 3 in the male and female/SPS-VEH group was significantly higher than in the male and female/Sham-VEH groups (P=0.0001) and fluoxetine decreased the expression of caspase 3 significantly in male and female/SPS-FLX groups compared to male and female /SPS-VEH groups (P=0.0001).

Also, fluoxetine significantly decreased the expression of caspase 3 in female/Sham-FLX groups compared to female/Sham-VEH (P=0.0001).

### Golgi-Cox staining

Figure 5 shows data on histological studies. In this Figure, tissue structure and dendritic branches in male and female SPS groups subjected to fluoxetine are shown. The density of the black color, which indicates dendritic branches and spine dendritic, is reduced in the male and female SPS groups. Fluoxetine administration improved this atrophy in the hippocampus. No significant difference was observed between male and female responsiveness to treatment.

## Discussion

This study was designed to examine the effects of fluoxetine administration (10 mg/kg/day) on serum IGF-1, spatial and recognition memory, apoptotic factors mRNA expression and tissue structure, dendritic branches of the hippocampus in adult male and female rats with SPS. We found that rats exposed to SPS had impaired location memory, and also showed reduced Serum IGF-1 and enhanced hippocampal proapoptotic markers, and decreased hippocampal dendritic branches. Fluoxetine alleviated SPS-induced behavioral, biochemical, and structural abnormalities and also apoptosis.

In the current study, SPS was utilized to induce PTSD. SPS as a PTSD animal model reveals a strong similarity to patients with PTSD in terms of behavior and physiology [35, 36]. This model is characterized by increased negative feedback inhibition of the hypothalamic-pituitary-adrenal axis, decreased fear extinction and hippocampal brain-derived neurotrophic factor (BDNF), increased anxiety behaviors and spatial and recognition memory impairment, and depression-like behaviors [4, 37, 38].

### SPS decreased memories index, effect of fluoxetine on memories index in male and female rats

Our study showed that male and female rats exposed to SPS had impaired recognition location memory. These results are consistent with another study showing that SPS impaired the spatial memory of rats in a radial maze task [31].

In this study, we observed SPS has a sex-dependent effect on recognition memory. In the male rats exposed to SPS, recognition memory showed a significant decrease compared to the sham group; however, in the female rats exposed to SPS, the decrease in the recognition memory was not significant and fluoxetine significantly increased DI in ORMT in both genders. The role of the serotonergic system in the recovery of the recognition memory after stress in male rats is known [39]. In confirming the results of our study, the study conducted by Bobbi Lee et al showed that SPS rats have a memory impairment [37]; and another study showed that the learning impairment in the ORMT was restored in stressed mice by acute fluoxetine treatment [40].

In the current study, SPS had no significant effect on DI in OLMT in male and female rats. Although fluoxetine increases DI in OLMT in male and female sham and SPS rats, this increase was not significant. Maybe

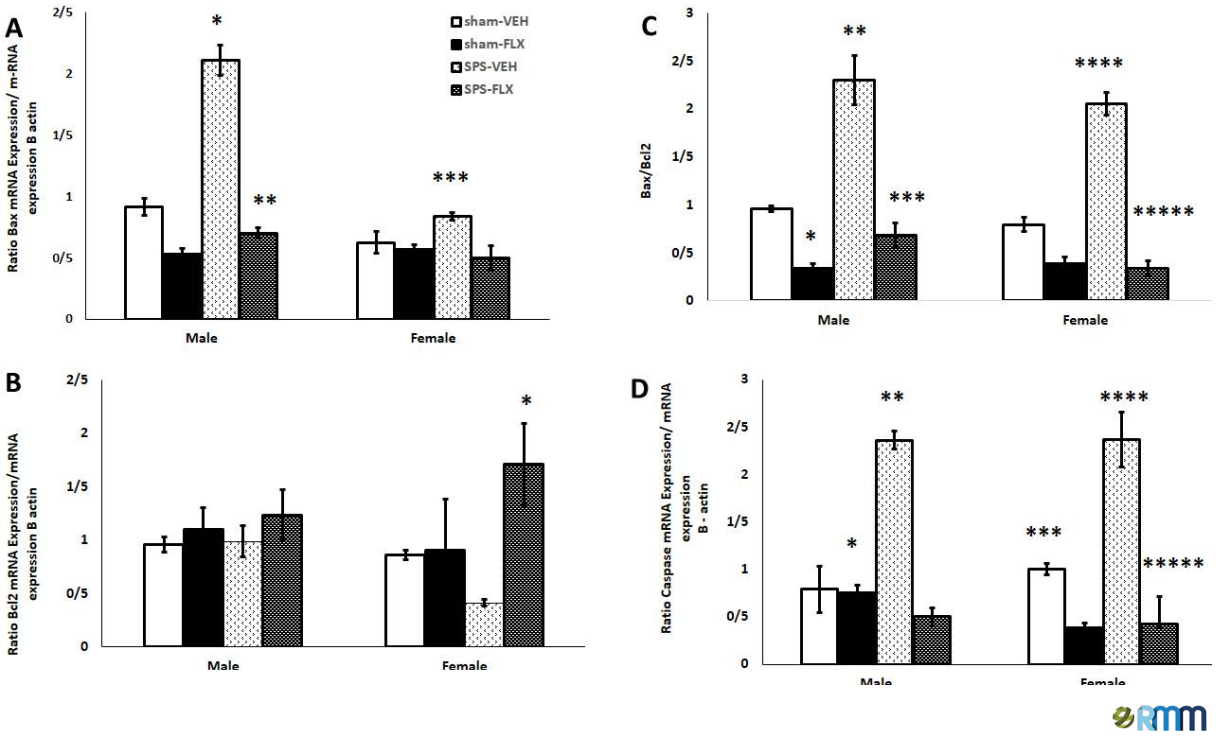



**Figure 4.** Expression of mRNA of apoptosis - related factors in hippocampal SPS rats subjected to fluoxetine treatment

A: Bax mRNA levels

* Male/SPS –VEH than Male/sham- VEH (P = 0.0001)

** Male/SPS –FLX than Male/ SPS - VEH (P = 0.0001)

**** Male /SPS –VEH than Female /SPS –VEH (P = 0.0001)

B: BCL-2 mRNA levels

* Female/SPS –FLX than Female/ SPS –VEH (P = 0.0001)

C: Ratio BAX/BCL-2

* Male/Sham –VEH than Male /sham- FLX (P = 0.006)

** Male/SPS –VEH than Male/sham- VEH (P = 0.0001)

*** Male/SPS –FLX than Male/ SPS - VEH (P = 0.0001)

**** Female/SPS –VEH than Female/sham- VEH (P = 0.0001)

*****Female/SPS –FLX than Female/ SPS - VEH (P = 0.0001)

D: Caspase-3 mRNA levels

*Male/SPS –VEH than Male/sham- VEH (P = 0.0001)

** Male/SPS –FLX than Male/ SPS - VEH (P = 0.0001)

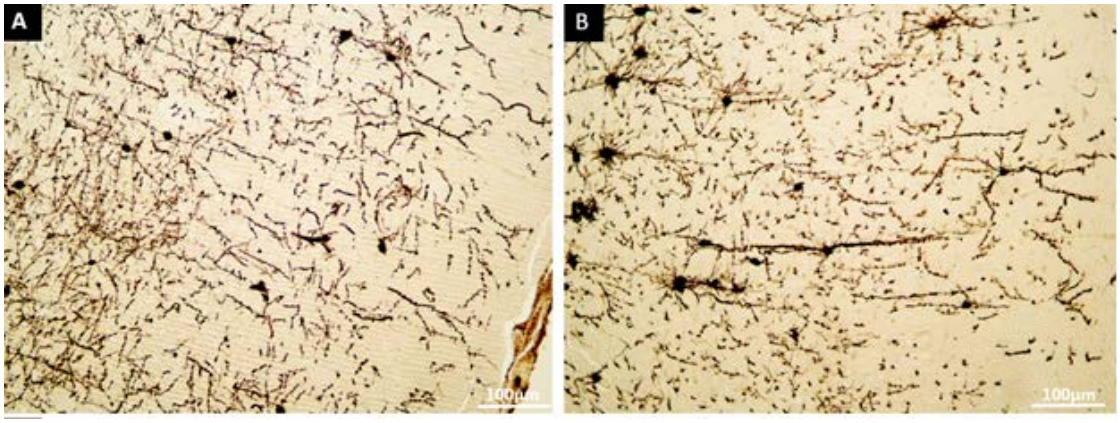
*** Female/Sham –VEH than Female /sham- FLX (P = 0.0001)

**** Female/SPS –VEH than Female/sham- VEH (P = 0.0001)

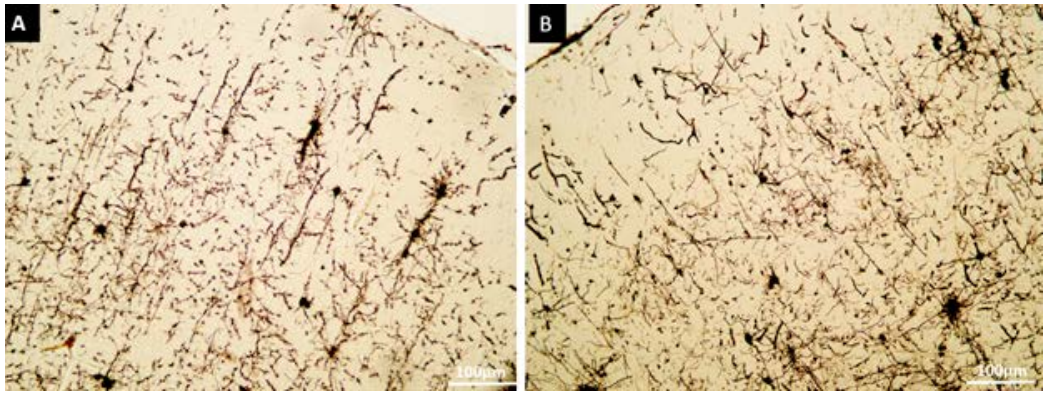
***** Female/SPS –FLX than Female/ SPS - VEH (P = 0.0001)

the dose of the drug used or the method of administering the drug or the duration of its effect has led to a significant lack of response in both genders.

Various studies have also recommended the use of antidepressants to improve spatial memory damaged by stress. Tianeptine as an antidepressant stops damage to spatial memory caused by predator stress and increases long-term memory by increasing the physiological function of the hippocampus, and increased synaptic plasticity in the central area 1 (CA1) region [41] and dendritic deformation block in the different hippocampus regions [42]. It has also been observed that the acute reduction of central serotonin levels damages object-related memory in adult rats [43] which refers to the crucial role of serotonin in the formation of spatial memory. Also, fluoxetine significantly increases neurogenesis, spine density of pyramidal neurons, and cognitive functions in animal




Data on histological studies on tissue structure and dendritic branches showed in female SPS group subjected to fluoxetine
A: Female rats exposed to SPS
B: Female rats exposed to SPS subjected to fluoxetine




Data on histological studies on tissue structure and dendritic branches showed in male SPS group subjected to fluoxetine A:
A: Male rats exposed to SPS
B: Male rats exposed to SPS subjected to fluoxetine

**Figure 5.** Golgi-Cox Staining in female and male rats

models of prefrontal cortex damage, and it is suggested that the up-regulation of BDNF is a possible mechanism underlying fluoxetine effects on dendritic spine plasticity in the prefrontal cortex [44, 45]. Furthermore, contrasting results are found regarding the effects of antidepressants on cognition and spine densities [46], also emotional memory deficits have been shown with acute fluoxetine treatment in healthy individual volunteers [47]. These differences may be related to differences in the animal models, duration and dose of treatment, types of laboratory animals, gonadal hormones, and types of behavioral tests used in these studies. The results of some studies showed the sex-dependent effects of fluoxetine. Treatment with fluoxetine improves passive avoidance, learning and memory, and prefrontal BDNF levels in several mental illnesses [48]. The results of other studies showed that females in reproductive ages have a better response to fluoxetine than men and during menopause, females metabolize fluoxetine better than males, therefore females in this period respond to a higher dose of fluoxetine than males [49]. Still, no complete consensus exists on gender differences in treatment response to an antidepressant. Some human studies have shown that men experience a better therapeutic response to tricyclic antidepressants (TCAs) than women, and other studies showed that women respond better to SSRI treatment than men. The difference in the effect of chronic stress on the density of hippocampal dendritic branches has also been shown in male and female rodents [50]. However, in the current study, gender differences in dendritic density and therapeutic response to fluoxetine were not observed. In the present study, we found that the serum IGF-1 in female/sham-FLX rats is higher than in male/sham-FLX rats. However, no significant difference was observed in the levels of serum IGF-1 between male and female/SPS-FLX groups.

Although previous studies showed gender differences in response to stress [51] and gonadal hormones play an essential role in the response to trauma [52], we did not observe significant differences in object and recognition memory between SPS-FLX male and female groups. Based on the results in male and female rats exposed to SPS, SPS probably leads to the inactivation of the neural pathways related to gonadal hormones and stress which means that no gender difference is observed in response to the fluoxetine.

### SPS decreased serum insulin-like growth factor 1 (IGF-1), effect of fluoxetine on serum IGF-1 in male and female rats

The present study showed that SPS can significantly reduce serum IG-1 levels in male and female rats, these results are consistent with previous studies in animal models of PTSD and prenatal and postnatal stressed rats, IGF-1 expression and IGF-1R phosphorylation decreased in the hippocampus, frontal cortex, and olfactory bulb, which is associated with depressive-like behaviors; also, intracerebroventricular administration of IGF-1 in stressed animals reversed these effects [53, 54].

It has been demonstrated that most patients with PTSD suffer social and behavioral abnormalities due to hippocampal atrophy in these patients [55]. One recent study showed that IGF-1 as a neuroactive hormone affects vulnerability to stress [56]. Also, the regulatory role of IGF-I on mood and coping strategies in stressful situations was recognized [57, 58]. A significant relationship is observed between IGF-1 levels and anxiety and cognitive disturbances. It has indicated an essential role of IGF-1 in psychiatric disease symptoms [59].

IGF-1 levels are higher in people after a head injury to protect and remove head injury [60]. In contrast to our results, another study has shown that in some diseases, such as major disorder or obsessive-compulsive disorder, IGF-1 levels are significantly increased in the brain compared to the control group [61]. Nakajima et al also discovered that chronic stress leads to a high expression of IGF-1 in the brain cortex and liver of rats. Therefore, it has been suggested that IGF-1 is involved in the anti-stress mechanism in the body [62].

The data revealed that the level of serum IGF-1 increased significantly in the female/sham-FLX group compared to sham-VEH, but in the male and female rats exposed to SPS groups, although FLX could increase IGF-1 levels, this amount could not lead to a significant difference. Perhaps the use of fluoxetine with a higher dose or a longer treatment period or the systemic injection caused a significant increase in the serum IGF-1 in SPs groups.

Fluoxetine is a selective serotonin reuptake inhibitor that exerts its therapeutic effects, by promoting neuroplasticity in the prefrontal cortex and hippocampus through increased BDNF/tropomyosin-related receptor kinase B signaling and the IGF-1 system [63]. Some studies have shown that fluoxetine can increase synaptic plasticity and neurogenesis in the amygdala and hippocampus of mice through BDNF and also relieve fear [64]. Infusion of fluoxetine or its administration in drinking water in mice decreases the symptoms of PTSD [65]. Ovarian steroids affect brain regions involved in the modulation of mood and behavior, and secretion fluctuations in these hormones can modify brain neurochemistry, indicating the pivotal role of sex steroids in controlling mood and behavioral disorders [66].

After trauma, it has been recognized that PTSD prevalence in females tends to be more severe than in men and they also develop stronger PTSD symptoms [52, 67]. The study conducted by Sakhaie et al showed that chronic fluoxetine treatment has sex-dependent effects on passive avoidance, memory, and BDNF mRNA expression, but in the pain threshold, fluoxetine treatment has no sex-dependent effects [48]. Other studies have also shown sex differences in the effects of antidepressants and clinical manifestations of many psychiatric diseases [26]. Sex hormones in females affect pharmacokinetics and the efficacy of antidepressants [68].

In our study, no significant difference was observed between male and female SPS groups, but a significant difference between male and female sham-FLX groups was observed.

Fluoxetine with a dose of 10 mg/kg caused a significant increase in IGF-1 in the female-sham group compared to the male-sham group.

Maybe this result is related to the interaction of these two systems together in females because the co-localization of the serotonergic and estrogenic systems supports the interactions between the two systems [69]. Sex hormones are associated with different efficacy of antidepressant drugs. Animal studies have confirmed an enhanced effect of SSRIs treatment following estradiol administration [70].

Other studies have shown that the hippocampus and cerebral cortex, which are involved in the control of both emotional and cognitive behaviors, are influenced by fluoxetine [71]. Valluzzi reported that hippocampal and non-hippocampal-dependent memories were also affected by fluoxetine [72].

## SPS increases expression of apoptosis-related markers: Beneficial effect of fluoxetine

Prevention of cell-programmed death may be caused by various molecular mechanisms, such as neurotrophins, and expression adjustment of apoptotic regulators [11]. The expression of IGF-1 is high in the developing brain tissue, decreases after adulthood, and increases when the adult brain suffers from internal and external environmental stimuli or injuries [55]. Despite several psychophysiological differences in the expression of psychiatric disorders between males and females, still, no complete agreement exists on the responsiveness of both sexes to treatment. Some studies have shown that, although differences are observed in depression symptoms and severity in males and females, they do not differ in mindedness and do not show any difference in the treatment [73].

However, many studies have been conducted in this field and they have tried to accommodate differences in medication, dose, regimen, compliance, antidepressant type, and consumption patterns. They showed a significantly greater treatment response for males than females for the tricyclic antidepressant (TCA) [74]. Some studies also failed to show reliable evidence of a difference between the women and men's response to antidepressant treatments [75]. It has been demonstrated that the variables involved in the development of chronic PTSD can affect treatment response. Hypothalamic-pituitary-adrenal (HPA) axis functional damage is the source of many injuries in PTSD, and biological, interpersonal, intrapersonal, and social origin factors affect HPA axis functioning. This influence is essential when considering interventions for trauma-related disorders between men and women. Genetic and medical imaging studies are used to investigate gender differences in treatment response to pharmacological and psychosocial interventions to treat anxiety disorders [76]. Some studies have also suggested further studies with larger sample sizes and more biological factors on different responses to treatment [77, 78]. Apoptosis or programmed cell death has an essential role in synaptic plasticity and alterations in size and function in some brain areas in patients with PTSD [79, 80]. Caspase activation as an apoptotic-related factor induces dendritic pruning and dendrite degeneration [81]. It has been demonstrated that apoptosis can be induced by three pathways, mitochondria [82], a dependent pathway death receptor and endoplasmic reticulum pathway [80]. In PTSD, abnormal apoptosis was observed in certain brain regions, including the hippocampus [83, 84], the amygdala [81, 85], and the medial prefrontal cortex [86], which are closely associated with abnormality in emotion and cognition [80]. This study, like our previous studies [28, 29, 83], showed SPS induces apoptosis in the hippocampus in both sexes. In the current study, the expression of Bax mRNA as a proapoptotic factor increased in the SPS group in both sexes, but this increase was significant only in males. Male rats exposed to SPS treated with fluoxetine showed a significant decrease in the expression of this factor, but in female rats, this decrease was not significant and may be due to the estrous cycle in females. Another pro-apoptotic factor is caspase 3, which in our study showed a significant increase in the SPS group in both sexes and a decrease in the rats exposed to SPS that were treated with fluoxetine. The results of our study are consistent with the results of Yang Yang's study [87]. To explain the ratio of proapoptotic to antiappoptotic factors, we used the ratio of Bax to Bcl-2. SPS caused a significant increase in the ratio of Bax to Bcl-2 in both male and female rats, and fluoxetine caused a significant decrease in the male and female rats exposed to SPS. Our results are consistent with HanShan's study which showed that fluoxetine attenuated IL-1β-induced apoptosis in cortical neurons [88], and Sharifi's study on Parkinsonian rats [89]. It has been demonstrated that most patients with PTSD suffer social and behavioral abnormalities due to hippocampal atrophy in these patients [55]. Another study showed that fluoxetine can improve impairments of cognitive functions and hippocampal synaptic plasticity in an animal model of Down syndrome [90]. Fluoxetine has been shown to have different neurological and cognitive effects on the nervous system through augmentation of BDNF in the nervous system [91] and can increase the proliferation of neurons and neurogenesis in the central nervous system (CNS) [22].

## SPS decreased dendritic branches in hippocampus: Beneficial effect of fluoxetine

In this qualitative study, we observed SPS-induced loss of dendritic branches in hippocampal neurons, consistent with other studies showing that chronic restrained stress promotes microglial cell activation and loss of dendritic spines in the medial prefrontal cortex and hippocampus [92, 93], and this impairs on behaviors, such as retrieval or extinction of conditioned fear and working memory [94]. The present study showed that the use of chronic fluoxetine can restore the reduction in hippocampal dendritic branches in male and female rats exposed to SPS, and no significant differences were observed between

the male and female groups. In confirmation of our study, another study on PTSD animal models showed that chronic treatment with fluoxetine can reduce the abnormalities induced by stress in sensitized fear behavior, hippocampal synaptic proteins, inflammatory responses, anxiety responses, HPA axis activity, hippocampal BDNF, and apoptosis biomarkers [95].

Despite the various human studies that have been conducted on gender differences in response to antidepressant treatment, still, no consensus exists on whether sex-related differences are observed in antidepressant treatment responsiveness. New data exists regarding sex differences in antidepressant treatment based on differences in antidepressant metabolism, absorption, distribution, and elimination.

## Conclusion

The results showed that male and female rats with PTSD have reduced levels of serum IGF1, impaired spatial memory in a recognition location memory task, enhanced apoptotic-related factors expression in the hippocampus, and decreased hippocampal dendritic branches, and fluoxetine treatment (10 mg/kg -28 days) alleviated these abnormalities in male and female rats exposed to SPS; however, fluoxetine had no sex-dependent effects on IGf-1 serum level and location and recognition memory, hippocampal apoptotic markers, and dendritic branches in the hippocampus.

### Limitations

This study had limitations. We did not determine the phase of estrous cycles in female animals by vaginal smear because it is a stressful procedure. This is a crucial limitation of the present study since levels of sex hormones can influence responsiveness.

Also, we did not analyze the correlation between behavioral and molecular data due to the large number of tests. These limitations should be considered in future studies.

## Ethical Considerations

### Compliance with ethical guidelines

The experiments were approved by the Ethical Committee of Mazandaran University of Medical Sciences (IR.MAZUMS..REC.1399.9011 & IR.MAZUMS... REC.1397.1261). All experimental trials were conducted by the National Institutes of Health Guide for the Care and Use of Laboratory Animals

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### Authors contribution's

Study design and supervision: Sakineh Shafia; Laboratory experiments and data collection: Reza Eshaghi-Gorji, Sareh Rashidi, Sakineh Shafia, Fereshteh Talebpour Amiri, andMansoureh Mirzae; Data analysis: Sakineh Shafia and Moslem Mohammadi; Writing the manuscript: Sakineh Shafia and Moslem Mohammadi; Final approval: All authors.

### Conflict of interest

The authors declared no conflict of interest.

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