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Experimental Research Article

Regular exercise and enriched environment ameliorate neuroinflammation in pilocarpine-induced epileptic rats by antioxidant activity and NLRP3/caspase-1 pathway inhibition

Fatemeh Moradi, Mahsa Jazini, Hadi Feizi, Mahin Ganjkhani*

Department of Physiology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran

ABSTRACT

Introduction: As a chronic neurological disorder, epilepsy is affected by social stress, which is one of the numerous complications in societies. In addition to medication, enriched environment (EE) and exercise are among the complementary strategies in the treatment of epilepsy. Oxidative stress, which potentially can activate the inflammatory pathways, is one of the causes of this disorder. So, we tried to examine thoroughly the beneficial impacts of EE and exercise on neuroinflammation in epileptic rats.

Methods: Male Wistar rats were divided into five groups of twelve rats each, including: a control group, a group induced with pilocarpine to simulate epilepsy, an epileptic group subjected to social stress, an epileptic group placed in an enriched environment, and an epileptic group subjected to an exercise regimen. The impact of social stress, enriched environment, and exercise on oxidative stress biomarkers was investigated through TBARS spectrophotometric test and the gene expression of NLRP3, Caspase-1, IL18, and IL1β were evaluated through real-time PCR method.

Results: Epilepsy and social stress caused a reduction in superoxide dismutase (SOD) and glutathione peroxidase (GPx) ($p<0.05$). Moreover, they resulted in an enhancement of plasma malondialdehyde (MDA), NLRP3, Caspase-1, interleukin-18 (IL18), and IL1β gene expression ($p<0.05$). Exercise increased the GPx and diminished the expression of Caspase-1 and IL-18 inflammatory genes ($p<0.05$). Accordingly, EE enhanced the SOD and GPx antioxidant indicators and reduced proinflammatory gene expression.

Conclusion: In this research, social stress resulted in elevated levels of oxidative markers and upregulation of inflammatory gene expression. EE and regular exercise improved the situation.

Keywords:
Temporal lobe epilepsy
Social stress
Enriched environment
Exercise
NLRP3 pathway

Introduction

Epilepsy is a prevalent nervous system disorder mainly described by hyperexcitability of neurons, leading to recurrent seizures, and is known as a major public health problem (Anwar et al., 2020)Temporal lobe epilepsy (TLE) represents a common form of partial epilepsy that

* Corresponding author: Mahin Ganjkhani, mganjkhani@zums.ac.ir
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constitutes a substantial proportion of the global prevalence of epilepsy, and frequently necessitates surgical intervention, and can exhibit resistance to antiepileptic drugs (Pereira Dalio et al., 2022). Prior research has demonstrated that oxidative stress and persistent inflammation are significant factors in the pathogenesis of epilepsy (Parsons et al., 2022). In fact, excessive reactive oxygen species (ROS) production including malondialdehyde (MDA) and superoxide dismutase (SOD), play a significant role in the pathogenesis of epilepsy and subsequent neuronal death following seizures (Keloglan et al., 2023; Olowe et al., 2020).

Stress is one of the most common factors which elicits seizures in people with epilepsy (Novakova et al., 2013). Physiological stressors could trigger the inflammasome, which engages caspase-1, followed by proinflammatory cytokines secretion such as interleukin-18 (IL-18) and interleukin-1β (IL-1β) (Dong et al., 2020). Therefore, in its capacity as a principal intracellular sensor of cellular stress cues, the NLRP3 (Nod-like receptor family pyrin domain containing 3) inflammasome could potentially have a pivotal role in the development of epilepsy by exacerbating the inflammatory response (Wu et al., 2019). NLRP3 is an intracellular agent that can react to different danger signals. Subsequent to activation, NLRP3 triggers the development of the inflammasome as a multiprotein complex. In fact, NLRP3 interacts with adaptor protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) through its pyrin domain. Formerly, through its caspase recruitment domain (CARD), ASC interacts with caspase-1 and activates it, which completes the process of NLRP3 inflammasome assembly (Blevins et al., 2022).

Exogenous factors, such as environmental enrichment (EE) and physical activity (PA), have demonstrated a significant capacity to enhance recovery following seizure (Yang et al., 2016).

It appears that EE and PA may attenuate the severity of pathological changes in epilepsy through inducing neurogenesis and/or reducing proinflammatory cytokines such as tumor-necrosis-factor (TNF)-α and interleukin (IL) (Fabel et al., 2009; Vrinda et al., 2017a; Zeraati et al., 2021). Nevertheless, the effects of EE and PA preconditioning on antioxidant activity and the NLRP3 inflammasome pathway in epileptic models are not clearly understood. Therefore, in the current study, the effects of EE and PA on gene expression of NLRP3 inflammasome, caspase1, IL-1β, and IL-18 were evaluated in a pilocarpine-induced epilepsy model by real-time PCR.

Similarly, the likely effect of these interventions on serum levels of MDA, an indicator of oxidative damage, and SOD and glutathione peroxidase (GPx), antioxidant enzymes, was also elucidated.

Materials and Methods

Experimental animals

The assessment procedure was authorized by the Institutional Animal Care and Use Committee of Zanjan University of Medical Sciences (IR.ZUMS.REC.1397.204) and EU (86/609/EEC). In this study, 60 adult male Wistar rats (250–300 g) were maintained in a regulated environment situations including a fixed 12-hour light/dark cycle, a temperature of 22 ± 2 °C, and free access to food and water. Before experiment, animals were accommodated and then separated in 5 experimental groups (n=12) which included a control group, a group induced with pilocarpine to simulate epilepsy (Ep), an epileptic group subjected to social stress (Ep+SS), an epileptic group placed in an enriched environment (Ep+EE), and an epileptic group following an exercise regimen (Ep+Ex). The epileptic rats were exposed to their respective social stress conditions, enriched environments, or exercise routines for 30 days as presented in Table 1.

Pilocarpine model of temporal lobe epilepsy (TLE)

The TLE is like one of the greatest, widely known tools for the study of the human epileptic brain. In this model, systemic administration of pilocarpine induces cholinergic properties and recurrent seizures in rats, followed by the development of chronic epilepsy. Animals treated with pilocarpine commonly exhibit behavioral alterations and partial seizures (Leroy et al., 2003). The animal epilepsy model was carried out by an intraperitoneal pilocarpine injection as defined before (Moradi et al., 2019). Briefly, 30 minutes prior to pilocarpine administration (350–400 mg/kg, i.p), animals received methyl scopolamine bromide (1 mg/kg s.c.) to minimize the side effects of pilocarpine hydrochloride. One hour after the primary epileptic occurrence, each affected rat received an injection of diazepam (2.5 mg/kg). Behavioral scoring determined by Racine's scale, animals that acquired 4,5 were chosen for the research (Racine 1972). Following seventeen days, rats were sacrificed

TABLE 1: Experimental grouping: groups and the animal-directed behaviors

Group	Exercise	Social stress	Enriched environment	Saline injection	Pilocarpine injection
	30 days of treadmill	30 days exchange cagemate	30 days special cages		
control	-	-	-	+	-
Ep	-	-	-	-	+
Ep+Ex	+	-	-	-	+
Ep +EE	-	-	+	-	+
Ep+SS	-	+	-	-	+

TABLE 2: Training program of 4 4-lane animal treadmill

Session (days)	speed (meter/ minute)	Slope (degree)	Duration (minutes)
1-7	24	0	35
8-14	24	5	40
15-21	27	10	45
22-30	30	15	50

for gene expression analysis (Moradi et al., 2019). According to previous findings, histological and gene expression changes in the hippocampus start at the time of the 2nd and 3rd weeks after the beginning of epileptic seizures (Abdanipour et al., 2011).

Enriched environment and social stress model

EE rats were commonly housed in groups of 6 per cage. In the cages (120× 80 × 100 cm), there were different types of objects (e.g., chains, boxes, ladders, metal barrels, etc.) which were altered daily. The EE manipulation was done for 30 days, 6 hours per day (9:00 am- 3:00 pm). To induce social stress, 2 classes of rats were housed in isolated cages (6 rats per cage). Animals in each group were housed with the same cagemates for at least seven days to set up social dominance within the group. Following this period, psychosocial stress was induced by randomly transferring two rats between cages every other day for 30 days. This exchange disturbs the social dominancy, obligating subjects to repeatedly accommodate to novel tense situations (Atrooz et al., 2021).

Workout program

Every rat in the exercise groups was skilled to run on a treadmill (13; 4-lane animal treadmill; IITC Life Science Inc., USA). They had one session each day, 5 days a week, for 30 days (Sayyah et al., 2022). The training program included the speed, slope, and duration of running on the treadmill are presented in Table 2.

As in our previous experiments, at the end of the study, animals were anesthetized with chloroform inhalation, and the brains were carefully removed from the cranium (Aguwa et al., 2020).

Samples were kept at –70 °C in order to RNA isolation. RNA extraction and semi -quantitative real-time PCR assay. According to the manufacturer's instructions, total RNA was extracted from hippocampal tissue using Trizol reagent (Invitrogen, Shanghai, China). One mg of RNA was used for cDNA synthesis by the reverse transcribed system (Takara, Japan). Reverse transcription polymerase chain reaction (RT-PCR) was carried out in a GeneMate thermal cycler (Jinge Instr, Hangzhou, China). RT- PCR was quantified by SYBR Green Master Mix (Takara Bio, Inc.) in a Rotor-Gene 6000 (Qiagene). PCR amplification was carried out using the following programs: denaturation of cDNA [35 cycles, 95ºC for 30 seconds], annealing [35 cycles: gradient (58-65 ºC) for 30 seconds], and extension [35 cycles: 72 ºC for 35 seconds]. Cycle threshold (Ct) values were obtained in triplicate for each sample, and the average was calculated. Gene expression levels were normalized to β-actin, which served as the housekeeping gene. The relative expression of IL-18, NLRP3, and IL-1β was determined using the 2_ΔΔCT method. The particular primer sequences are listed in Table 3.

TABLE 3: The sequence of primers utilized in Real-Time PCR.

Genes	Primer sequences PCR	Product size	Tm
NLRP3	FP: TTCTCTGCATGCCGTATCTGG	88	61
	RP: TCATGTCCTGAGCCATGGAAG		61
Caspase 1	FP: ATGGAAAAGGCACGAGACCTG	134	61
	RP: GCTGATGGACCTGACTGAAGC		63
IL-18	FP: TATCGACCGAACAGCCAACG	90	60
	RP: GATAGGGTCACAGCCAGTCC		63
IL-1β	FP: CAGCTTTCGACAGTGAGGAGA	138	61
	RP: TGTCGAGATGCTGCTGTGAG		60
Beta-actin	FP: GCTCTATCCTGGCCTCACTG	136	63
	RP: GAAAGGGTGTAAAACGCAGCTC		62

Thiol groups analyze

Whole serum thiol level or sulfhydryl groups (SH) were quantified with the protocol previously used by Elmman (Costa et al., 2006) and later improved by Hu (Hu 1994). Thiols interact with 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), making an extremely colored anion with the highest peak at 412 ηm. According to the protocol, 25 µL of serum was mixed with 1 mL of Tris-EDTA buffer, and absorbance was measured at 412 nm (A1). Subsequently, DTNB (10 mmol/L in pure methanol) was added to the solution. After a 15-minute incubation time at room temperature, the absorptivity was reassessed (A2) in conjunction with a DTNB as the control (B). Utilizing reduced glutathione as the standard for sulfhydryl groups, the concentration of sulfhydryl groups was quantified and reported in mmol/L (Costa et al., 2006).

Malondialdehyde assay

Malondialdehyde (MDA) serum concentration was measured by Thiobarbituric acid reactive substances (TBARS) spectrophotometric test (Atmaca 2004). In the manual procedure, a volume of 200 µL of serum was combined with 2 mL of a solution containing 15% (w/v) trichloroacetic acid, 0.38% (w/v) thiobarbituric acid, and 0.25N hydrochloric acid. A red complex of TBARS was formed when it reacted with MDA in the supernatant. The absorbance of the TBARS complex was measured at 532 nm (Aliabadi et al., 2016). Serum total MDA was quantified by comparing the absorbance of test samples to that of standard samples, with MDA serving as the standard. The outcomes were expressed as µmol TBARs/L.

Superoxide dismutase activity assay

Briefly, the plasma supernatants were incubated in potassium phosphate buffer with xanthine and xanthine oxidase for 40 min (37°C, pH=7.8). Thereafter, nitroblue tetrazolium (NBT) was added. The intensity of blue formazan was quantified by spectrophotometry at 540 nm. The quantity of protein that prevented NBT reduction to 50% of the maximum was considered as 1 nitrite unit of SOD activity (Aliabadi et al., 2016).

Statistics

Analytical studies were done by SPSS (version 15.0). All the data are obtainable as mean ± standard error of mean (SEM) through independent tests that were repeated five times. One-way ANOVA followed by Tukey's post hoc was utilized for data comparison between the groups. The significance level was set at $P \leq 0.05$. The gene expression fold changes were considered with Rest software (version 2009).

Results

Oxidative parameters of GPx, SOD and MDA

As shown in Figure 1, the glutathione peroxidase activity, which is an antioxidant enzyme, in exercise (0.27±0.07) and enriched environment (0.27±0.07) groups was significantly enhanced compared with the epilepsy group (0.03± 0.004) ($P< 0.05$). Accordingly, superoxide dismutase activity, which is also an antioxidant enzyme, in the enriched environment group meaningfully elevated (31±2.8) in compared to the epilepsy group (25±1.64) ($P < 0.05$) (Figure 2). Alternatively, as indicated in Figure 3, there was a substantial rise in malond-

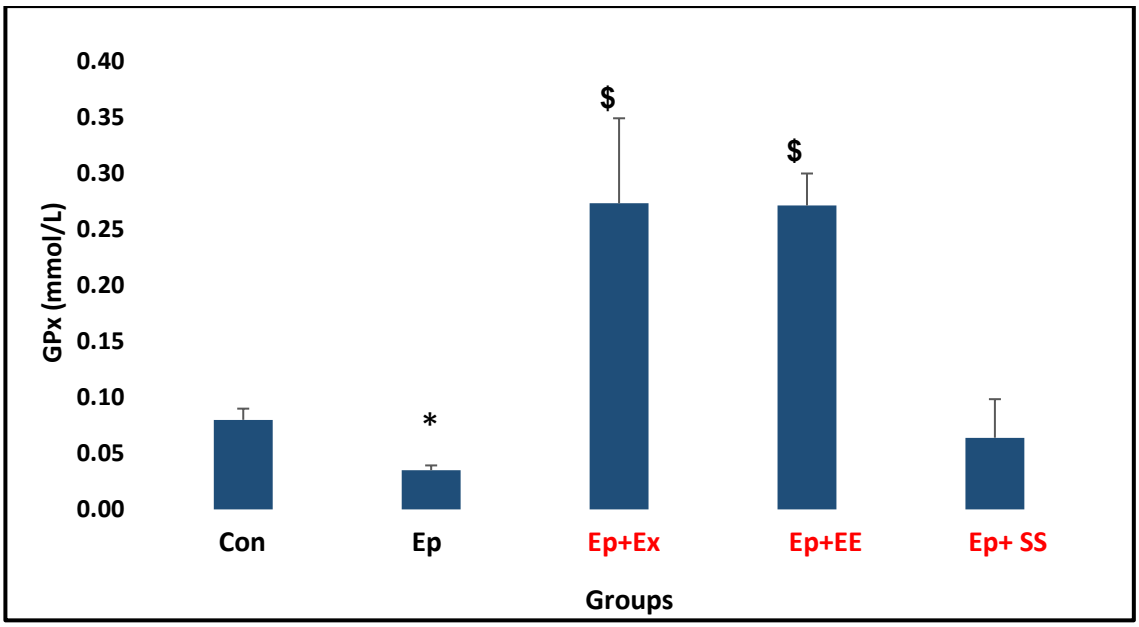

FIGURE 1. Effect of exercise (Ex), enriched environment (EE) and social stress (SS) on glutation peroxidase (GPx) activity in the serum of epileptic rats. The values are presented as means ± SEM in mmol/L (n=12). One-way ANOVA followed by Tukey's post hoc was utilized for data comparison between the groups. *P < 0.05 compared to the control group. $P < 0.05 for Ep+Ex and Ep+EE groups compared with Ep group. Groups: Control (con), epilepsy (Ep), exercise (Ex), enriched environment (EE), and social stress (SS).

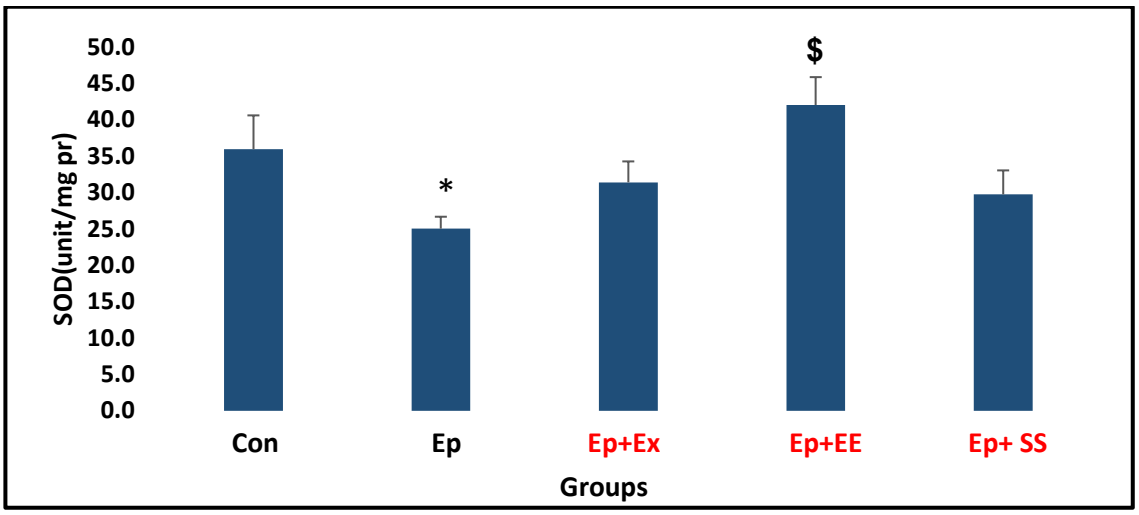

FIGURE 2. Effect of exercise (Ex), enriched environment (EE) and social stress (SS) on superoxide dismutase (SOD) activity in the serum of epileptic rats. The values are presented as means ± SEM in unit /mg pr (n=12). One-way ANOVA followed by Tukey's post hoc was utilized for data comparison between the groups. * P< 0.05 compared with the control group. $ P < 0.05 for the Ep+EE group compared with Ep alone. Groups: Control (con), epilepsy (Ep), exercise (Ex), enriched environment (EE), and social stress (SS).

ialdehyde function (an indicator of oxidative damage) in the animals of the Ep group (0.95±0.08) by comparison with the control group (0.29±0.08) (P < 0.05).

Quantitative Polymerase Chain Reaction (qPCR)

NLRP3

As presented in Figure 4, the mean relative expression of NLRP3 presented a remarkable increase in epilepsy (p<0.05) in comparison with the control group. But, the expression of this gene in epilepsy+ exercise and epilepsy+ enriched environment groups significantly decreased in comparison with the epilepsy group (p<0.05).

Caspase-1

The mean relative expression of Caspase-1 significantly increased in the epilepsy group compared with the control group (p<0.05). In contrast, there was a significant decrease in Caspase-1 gene expression in the

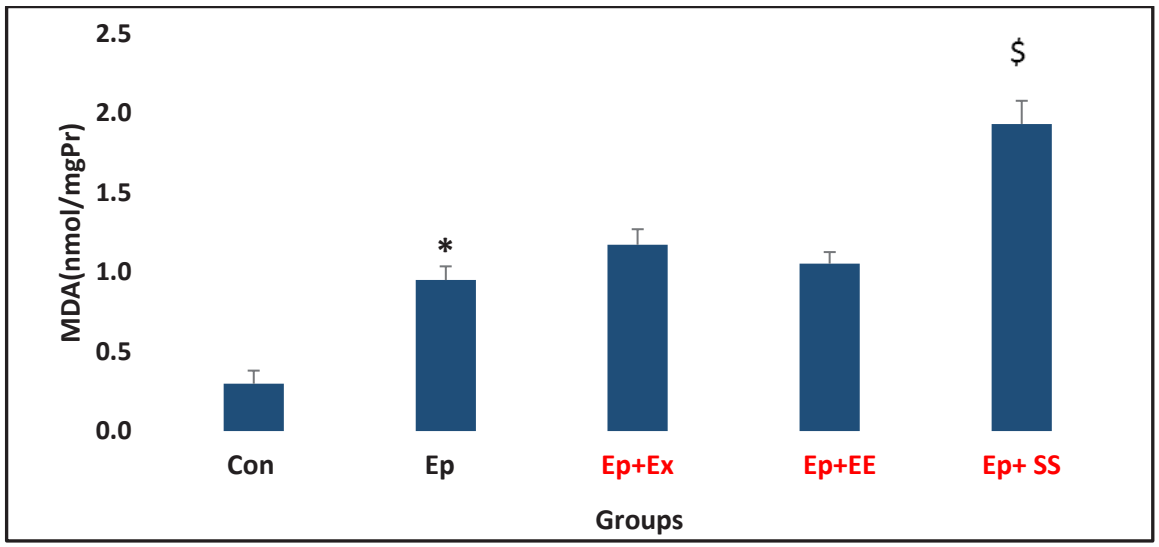

FIGURE 3. Effect of exercise (Ex), enriched environment (EE) and social stress (SS) on malondialdehde(MAD) activity in the serum of epileptic rats. The values are presented as means ± SEM in unit /mg pr (n=12). One-way ANOVA followed by Tukey's post hoc was utilized for data comparison between the groups. * P< 0.05 compared with the control group. $ P < 0.05 for the Ep+SS group compared with Ep alone. Groups: Control (con), epilepsy (Ep), exercise (Ex), enriched environment (EE), and social stress (SS).

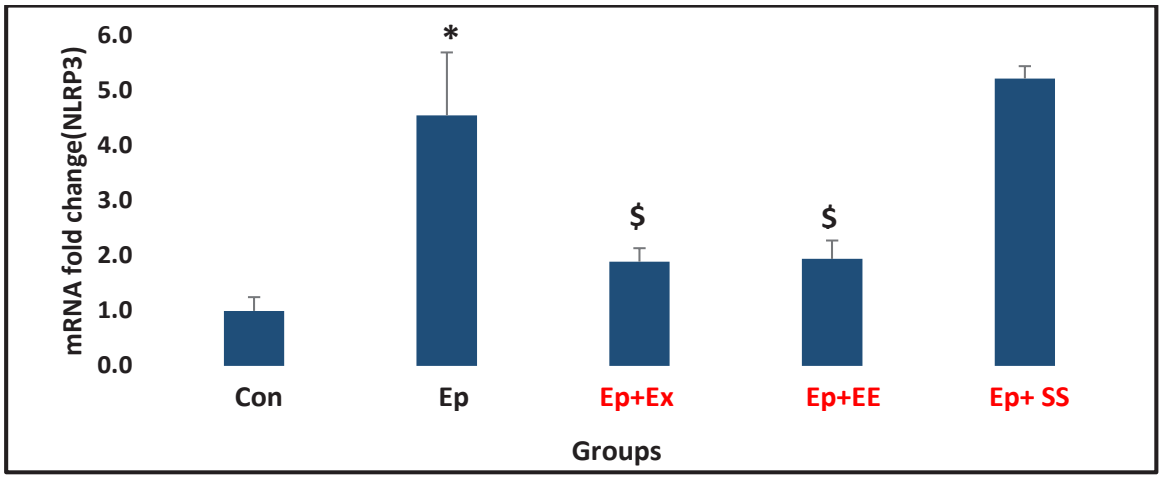

FIGURE 4. The mean relative expression of the NLRP3 gene in the conditions of exercise (Ex), enriched environment (EE) and social stress (SS) of epileptic rats. The values are presented as means ± SEM. One-way ANOVA followed by Tukey's post hoc was utilized for data comparison between the groups. * P< 0.05 compared with the control group. $ P < 0.05 for the Ep+Ex and Ep+EE groups compared with Ep group. Groups: Control (con), epilepsy (Ep), exercise (Ex), enriched environment (EE), and social stress (SS).

epilepsy + exercise and epilepsy + enriched environment groups compared to the epilepsy group ($p<0.05$) (Figure 5).

IL-1β

As indicated in Figure 6, the mean relative expression of IL-1β presented a remarkable increase in the epilepsy group compared with the control group. There was a significant reduction in epilepsy + enriched environment group by comparison with the epilepsy group ($p<0.05$), whilst this factor was increased in epilepsy+ social stress group compared with epilepsy ($p<0.05$).

IL-18

The IL-18 mean relative expression showed a significant increase in the epilepsy group compared with the control group ($p<0.05$). Instead, in epilepsy+ exercise and epilepsy+ enriched environment groups, the interventions on epileptic animals led to a significant reduction in IL-18 gene expression in comparison with the epilepsy group ($p<0.05$) (Figure 7).

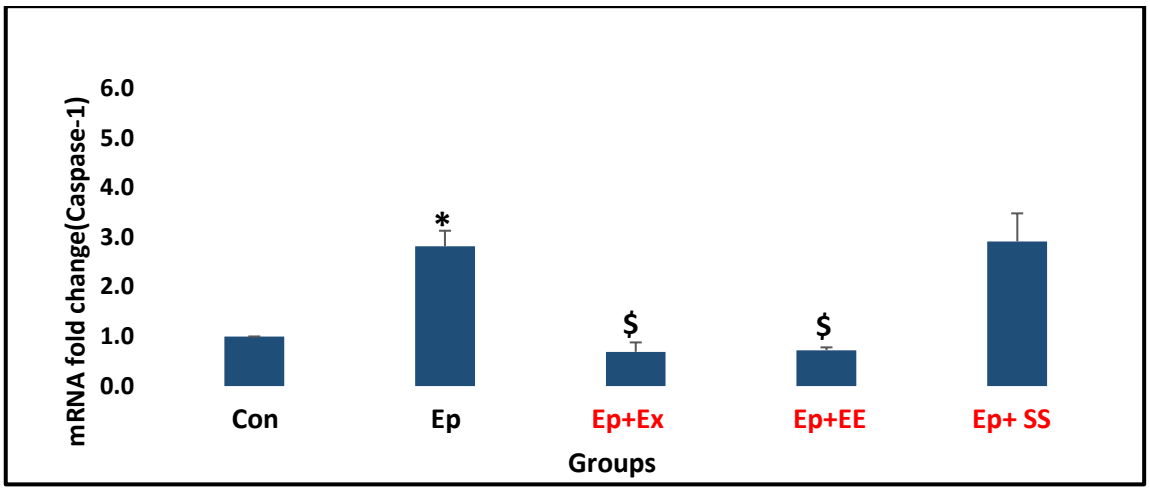

FIGURE 5. The mean relative expression of the Caspase-1 gene in the conditions of exercise (Ex), enriched environment (EE) and social stress (SS) of epileptic rats. The values are presented as means ± SEM. One-way ANOVA followed by Tukey's post hoc was utilized for data comparison between the groups. * P< 0.05 compared with the control group. $ P < 0.05 for the Ep+Ex and Ep+EE groups compared with Ep group. Groups: Control (con), epilepsy (Ep), exercise (Ex), enriched environment (EE), and social stress (SS).

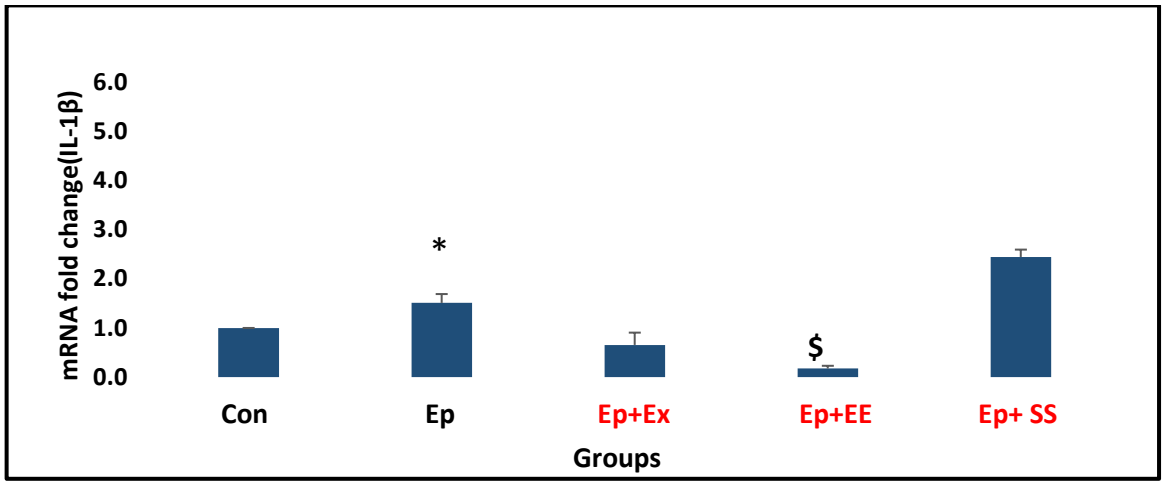

FIGURE 6. The mean relative expression of the IL-1ß gene in the conditions of exercise (Ex), enriched environment (EE) and social stress (SS) of epileptic rats. The values are presented as means ± SEM. One-way ANOVA followed by Tukey's post hoc was utilized for data comparison between the groups. * P< 0.05 compared with the control group. $ P < 0.05 for the Ep+EE group compared with Ep group. Groups: Control (con), epilepsy (Ep), exercise (Ex), enriched environment (EE), and social stress (SS).

Discussion

In current research, we observed that social stress leads to an elevated in MDA as oxidative indicator and a decrease in SOD and GPx, as antioxidant indicators. These results are consistent with earlier studies. It seems that chronic stress triggers a cascade of oxidative reactions and neurodegenerative events in the temporal lobe that may be associated with increased neuronal circuit excitability in the brain, thus increasing epilepsy susceptibility (Zhu et al., 2017). An increase in oxidative stress indicators due to social stress was also observed in the hippocampus in other studies (Patki et al., 2013). A study by Yuhan Shao in 2015 showed that mice under social isolation encountered a reduction in the brain tissue antioxidant capacity after 8 weeks (Shao et al., 2015).

In this study, the enriched environment increased the SOD and GPx antioxidant indicators in the animals. According to a study by Auvergne et al., an enriched environment delays the spread of epilepsy (Auvergne et al., 2002). Hence, while exposed to EE, the susceptibility of animals to seizures is diminished (Korbey et al., 2008). Another study has shown that EE also defends counter kainite kainite-induced epilepsy (Young et al., 1999). Vrinda and colleagues in 2017 showed that exposure to EE reduces the number and duration of seizures

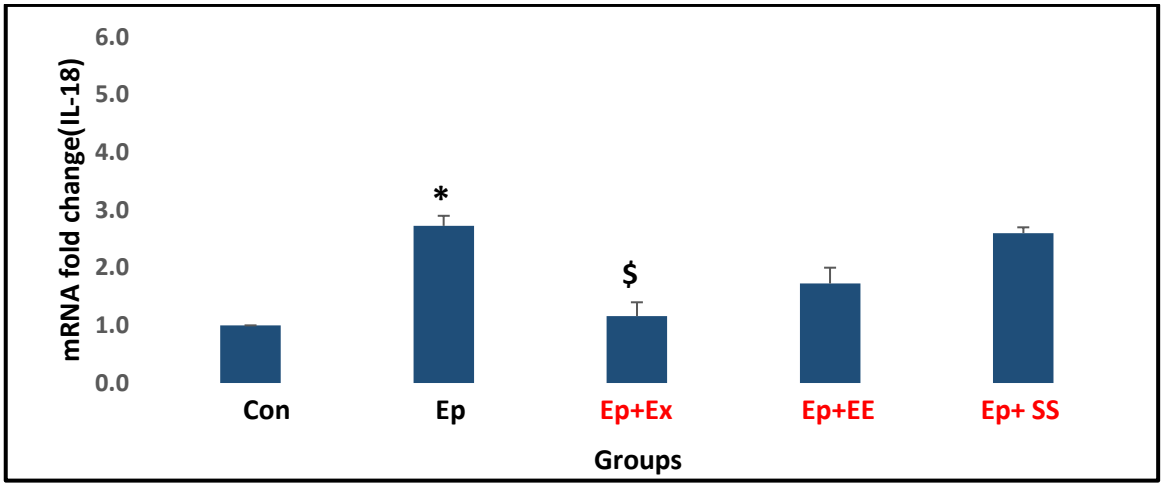

FIGURE 7. The mean relative expression of the IL-18 gene in the conditions of exercise (Ex), enriched environment (EE) and social stress (SS) of epileptic rats. The values are presented as means ± SEM. One-way ANOVA followed by Tukey's post hoc was utilized for data comparison between the groups. * P< 0.05 compared with the control group. $ P < 0.05 for the Ep+Ex group compared with Ep group. Groups: Control (con), epilepsy (Ep), exercise (Ex), enriched environment (EE), and social stress (SS).

in the laboratory model of TLE. As a result, EE also had an antidepressant effect in epileptic animals and eliminated hyperexcitability in the TLE model (Vrinda et al., 2017b). Studies have shown that EE can reduce oxidative damage in the nervous system, as a consequence, reinforcing the antioxidant defense process (Opii et al., 2008).

Furthermore, stress can induce ROS production and ATP, which are the key activators of NLRP3 inflammasome, leading to inflammation expansion (Coll et al., 2015; Iwata et al., 2013). A survey on the mechanism of depressive-like and anxiety-like behaviors showed that chronic stress increases extracellular ATP, Caspase-1, NLRP3 inflammasome formation, and IL-1β activation in the hippocampal region of rodents (Yue et al., 2017). Furthermore, chronic social stress increases levels of NLRP3, activated IL-1β, and Caspase-1 in the hippocampus and decreases NLRP3 inflammasome inhibitor levels (Pan et al., 2014). Therefore, it can reduce the activation level of inflammatory genes.

Consistent with our findings, EE reduced the activation of inflammatory genes Caspase-1 and IL-1β. A study on memory function found that EE could reduce overactivity of the hypothalamic pituitary adrenal stress response, as well as reduce oxidative damage by modulating inflammatory agents and increasing the antioxidant capacity (Nawaz et al., 2018). Results of a paper pointed out that EE could improve post-stroke cognitive disorders in rats through inhibiting oxidative stress and neuroinflammation. It also restored astrocyte activity and BDNF production in the hippocampus (Zhang et al., 2020). In line with previous studies, Chabry et al. demonstrated that enriched environment (EE) can inhibit the expression of proinflammatory genes, thereby exerting antidepressant effects (Chabry et al., 2015).

In this study, the exercise protocol employed was a combination of strength and endurance training, which increased in intensity and duration over time. The animals were required to run on a treadmill prior to entering the study process, thus ensuring that they did not receive a shock during the study. The aforementioned exercise protocol was based on numerous studies and yielded results indicating a reduction in oxidant factors and an increase in the power of variables. According to our results, exercise increased the antioxidant indicator GPx. These data convey that physical activity ameliorates oxidative stress damage in pilocarpine-induced epileptic rats. According to the Rambo et al. study, exercise reduces the incidence of seizures and seizure frequency in the chronic stage of this particular model, thus raising the seizure threshold (Arida et al., 1999; Rambo et al., 2009; Setkowicz and Mazur 2006). This finding is consistent with earlier studies showing the beneficial effect of continuous workout on the redox status of the rodent neural system after swimming and treadmill exercise (Coşkun et al., 2005; Jolitha et al., 2006; Servais et al., 2003). Furthermore, exercise had an essential role in the antioxidant enzymes activity (Holmes et al., 2015;

Kiran et al., 2004). Lee found that the SOD expression and catalase decreases in the kainic acid epilepsy model and that exercise pretreatment plays a neuroprotective role against the oxidative stress induced impairment. This protection was supported by inhibiting NO production and activating antioxidant enzymes, respectively. Thus, exercise has been proposed as an effective plan to prevent oxidative stress and, consequently, epilepsy (Yanpallewar et al., 2004; Yanpallewar et al., 2005).

As mentioned in the introduction, inflammation is one of the factors involved in the pathogenesis of epilepsy. Meanwhile, another protective effect of exercise could be due to its anti-inflammatory properties. In this regard, exercise can regulate circulatory concentrations of TNF-α and IL-6 in diabetic patients (Balducci et al., 2010; Nishida et al., 2014). NLRP3 level increases in most inflammatory diseases, and exercise with appropriate intensity can reduce this type of inflammasome (Khakroo Abkenar et al., 2019; Li et al., 2020; Liang et al., 2020). Studies on high-fat diet model mice have indicated that regular cardio workout decreases levels of IL-1β, Caspase-1, NLRP3, and IL-18 in the hippocampal formation of exercise-treated animals (Liang et al., 2020; Wang et al., 2016). Also, a study in 2018 indicated that by suppressing the activity of NLRP3 inflammasome, regular exercise can be an effective approach to protect against diet-induced vascular disorders. It is noteworthy that in the current study, a reduction in the expression of the inflammatory genes IL-18 and caspase-1 was observed in the epileptic rats that received the exercise treatment.

Conclusion

Taken together, epilepsy and social stress led to reduced antioxidant capacity in the brain. These conditions also increased the expression of pro-inflammatory genes such as NLRP3 inflammasome, IL-18, IL-1β, and caspase1. The protocols used in this study, which included exercise and EE, not only restored antioxidant levels but also restored the expression of inflammatory genes. Therefore, further investigation of such strategies should be a high priority, using both basic and clinical approaches, to facilitate the development of new approaches to prevent, delay, and treat epilepsy.

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Conflict of interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of the paper. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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