



## Research paper

# Erythropoietin attenuates locomotor and cognitive impairments in male rats subjected to physical and psychological stress

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## ABSTRACT

Physical and cognitive problems associated with stress are believed to result from stress-related damage to neurons involved in motor and cognitive control. In general, there are two types of stress, physical and psychological which both negatively impact neuronal function. Erythropoietin (EPO) has been shown to exert a neuroprotective effect in various models of physical brain injury; however, its actions on stress-related changes in behavior are unknown. The aim of the current study was to determine whether EPO ameliorated stress-induced locomotor and cognitive impairments, and to compare the effects of EPO on behavioral changes induced by the two different types of stressors. In this study, male Wistar rats were randomly divided into five groups and placed under physical or psychological stress for 10 consecutive days while erythropoietin was injected intraperitoneally (i.p.) every other day (500 U/kg/i.p.) 30 min before stress induction. Exploratory, anxiety-related behaviors, learning and memory were assessed by using open field, plus maze and Morris Water Maze (MWM) tests respectively. Our data showed physical and psychological stress induced dysfunction in locomotion, reduced explorative skills, heightened anxiety-like behavior and reduced memory, which could be partly reversed by EPO. We conclude that EPO reduces adverse effects of both psychological and physical stress, putatively through protection of locomotor and cognitive-controlling neurons vulnerable to the damaging effects of stress. However, future studies need to elucidate the neural mechanisms of the protective effects of EPO.

## 1. Introduction

In a biological system, stress is defined as any condition that seriously alters physiological/psychological homeostasis. Stress can be caused by emotional and physical stressors. Physical stressors directly affect the body but emotional stressors cause stress only through the information that reaches the brain without having a direct physical effect on the body (Hong et al., 2018). Whether a stressor physically contacts the body, or is processed through neural transmission, both types of stressors can result in serious physiological harm and cause disorders affecting various organ systems (Hong et al., 2018). In recent decades, the negative effects of stress on different aspects of cognitive and affective function have been recognized, and it is now clear that prolonged exposure to stress increases the risk of depression, anxiety

disorder and schizophrenia (Sapolsky et al., 2000; Razavinasab et al., 2020), as well as leads to adverse effects on learning, memory and age-related cognitive problems (Perna et al., 2016). The effects of stress on locomotor control has been less well studied, however, chronic stress was associated with severe alterations in motor behaviors in rats (Moreno-Martínez et al., 2022).

Effects of stress on locomotor and cognitive functions have been shown to be due to stress-induced neural atrophy and necrosis. Metabolic stress was shown to induce atrophy in the hippocampus characterized as debranching and shortening of the apical membrane (Magarinos et al., 1999; Hueston et al., 2017; Castelli et al., 2019). Stress was shown to result in loss of synapses in the motor cortex of mice which were associated with motor learning deficits (Gellner et al., 2022).

While reductions in stress can ameliorate neural damage, reducing

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stress is not often an easy option. Unfortunately, other than avoiding stress, there is no specific and predetermined treatment in order to decrease stress and its negative neuronal effects, thus new targets are mandated. In that regard, the erythropoietin (EPO) system is an interesting candidate as while EPO and its receptor are expressed in neurons and astrocytes (Juul and Yachnis, 1999), exogenously applied EPO can cross the blood brain barrier (BBB) and induces neuroprotective and neurotrophic effects in various experimental models of brain injury (Brines et al., 2000; Memisoglu et al., 2017). In addition, EPO results in enhancement of cognitive function in healthy animals (El-Kordi et al., 2009). These significant neuroprotective effects of EPO have resulted in use of EPO in clinical studies for treatment of neurocognitive disorders in patients with neurodegenerative or neuropsychiatric status including schizophrenic or multiple sclerosis (MS) (Ehrenreich et al., 2007; Ehrenreich et al., 2007).

To the best of our knowledge, there are no preclinical studies comparing and contrasting the effects of EPO treatment on motor and cognitive behavioral outcomes following physical and psychological stress. Therefore, we wished to evaluate the effects of EPO on locomotor and cognitive impairments induced by physical and psychological stress. For our studies, we used the communication stress box model to induce both physical and psychological stress. In one box, male rats were forced to swim, which is a physical stressor, and in the other box, rats were forced to observe those undergoing forced swimming which confers psychological stress (Nazeri et al., 2015; Razavinasab et al., 2020). The main aim of the current study was to evaluate effects of EPO in ameliorating impairments induced by two different modalities of stress.

## 2. Material and methods

### 2.1. Animal preparation

Male Wistar rats ( $n = 40$ , 12 weeks old), were purchased from the Neuropharmacology Institute and kept under standard conditions (temperature  $22 \pm 3$  °C and a 12-h light/dark cycle) with constant monitoring by a veterinarian. All protocols were approved by the Animal Ethics Committee of this institution [code: EC/KNRC/95–8].

### 2.2. Treatment

The rats received saline or EPO (500 U/kg) intraperitoneally every other day (Feere et al., 2015) 30 min before stress induction. EPO was provided as a gift from Pouyesh Darou product Company.

### 2.3. Experimental groups

Animals were randomly divided into five groups ( $n = 8$  for each group): sham group who received only intraperitoneal saline, physical stress group (Phy), psychological stress group (Psy), physical stress with erythropoietin group (Phy+EPO) and psychological stress with erythropoietin group (Psy+EPO).

### 2.4. Stress exposure procedure

Animals were exposed to different types of physical or psychological stress for 10 consecutive days. Two types of stress were induced by a communication stress box, which had 9 chambers (each chamber:  $25 * 30 * 45$  cm) separated from each other by a transparent Plexiglas that allows rats in different chambers to see each other. Five chambers which were to be used to physically stress rats was connected to an electric pump. The physical stress group received physical stress by being placed individually for 10 min in a chamber that had been filled with water ( $20 \pm 2$  °C) through the electrical pump so that animals had to swim in each chamber. Swim stress is a known stressor that has already been shown to elicit stress responses. After 10 min, the water was drained away and the protocol was repeated after 5 min rest for a total duration of one hour. At

the end of the experiment, animals were dried with a clean, dry towel and moved to their cages. The animals that received psychological stress were placed in other chambers which allowed them to watch the animals receiving physical stress. The chambers of the communication stress box which were to contain the psychological stress group were not connected to the pump (Nazeri et al., 2015; Nazeri et al., 2017).

### 2.5. Open field test

The open field test was used to measure locomotor activities and anxiety-like behaviors in order to understand effects of EPO on physical and psychological stress in animals. This apparatus consists of a square arena ( $90 * 90 * 30$  cm) made up of Plexiglas. The floor of the apparatus was divided into 16 squares and had two main peripheral and central squares in which animals were placed and parameters such as total distance moved [TDM, cm], mobility, immobility, velocity and duration were recorded by an automated video tracking system in 5 min periods (Aghaei et al., 2015).

### 2.6. Elevated plus maze

An elevated plus shaped maze (EPM) with two open arms ( $50 \text{ cm} \times 15 \text{ cm} \times 2 \text{ cm}$ ) and two enclosed arms ( $50 \text{ cm} \times 15 \text{ cm} \times 40 \text{ cm}$ ) was used to assess anxiety-like behaviors in rats. Animals were individually placed in the center of the maze facing an open arm and allowed to explore the maze for 5 min. TDM, velocity, number of entrances into the open and close arms and time spent in each arm were recorded by a video camera installed above the EPM. Behaviors such as total time spent in open arm, total time spent in the closed arm, and the ratio (%) (Ratio of open/total arm entries and ratio of close/total arm entries) were calculated as a standard anxiety index. In order to remove cues, between each trial, the maze was cleaned with 70% alcohol (Razavinasab et al., 2020).

### 2.7. Morris water maze

The Morris Water Maze (MWM) was used for measuring spatial learning and memory. The maze consisted of a black circular tank ( $140 \text{ cm}$  wide,  $45 \text{ cm}$  high) filled with water at a temperature of  $23 \pm 1$  °C. The tank was divided in four quadrants which incorporated visual cues. During the acquisition phase, animals were tested in three blocks. Each block contained four trials in which animals were placed in different locations (N, S, E or W). The inter-block interval was at least 30 min for each rat. In each trial, the rat had 60 s to find a platform that was submerged in 2 cm below the water surface placed in the north-east quadrant of the maze. Rats were allowed to stay at the platform for 15 s (location of platform did not change during this phase), and then they were transferred to the cage for 20–30 s, followed by a return to the water maze. In this phase, time and distance traveled to reach the platform were recorded by Ethovision software (Noldus Technology, Wageningen, the Netherlands). Two hours after the final trial which was in the third block, the platform was removed from the maze to obtain measurement of spatial memory. Animals were allowed to swim in the maze for 60 s and distance and time spent in the target quadrant was measured (Aghaei et al., 2015).

### 2.8. Statistical analysis

SPSS software (version 18) was used for the analysis of data. All data were expressed as mean  $\pm$  SEM.  $P < 0.05$  was considered statistically significant. One-way ANOVA followed by Tukey's post hoc analysis was used to compare the differences among experimental groups. Repeated measures ANOVA was performed on the learning phase of the MWM.

### 3. Results

#### 3.1. The effect of stress and EPO administration on locomotor activity and anxiety-related behaviors

The open field is designed to evaluate anxiety and locomotor activities. Our results showed unsurprisingly that both physical and psychological stress altered some parameters in the open field. Locomotion was measured as total distance moved (TDM) as shown in Fig. 1A and TDM was significantly smaller in groups exposed to physical and psychological stress compared with the sham group ( $p < 0.05$ ). EPO was found to ameliorate the stress-induced effect on TDM, as animals treated with EPO showed a greater TDM in both Phy+EPO and Psy+EPO groups, and there was no significant difference between the EPO treated groups and sham. As illustrated in Fig. 1B, the duration of immobility was very similar across all groups, and there was no significant difference in this parameter.

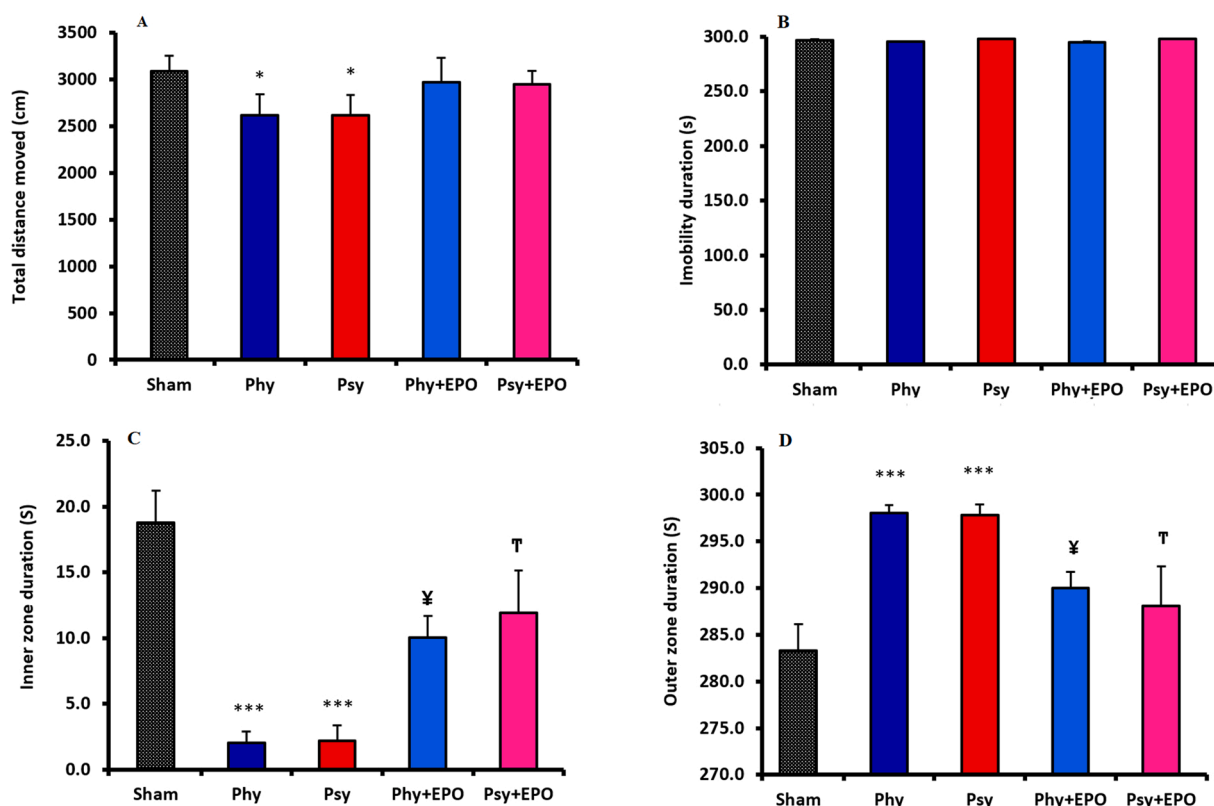
Time spent exploring in the inner zone was influenced by stress, which was expected as anxiety is associated with a reduced willingness to explore the inner zone. Duration of time spent in the inner zone was lower in Phy and Psy groups compared with the sham group ( $p < 0.001$ ; Fig. 1C). There was a significant difference in this parameter in the groups treated with EPO as the Phy+EPO and Psy+EPO groups showed a significantly longer duration in the inner zone than the Phy and Psy stressed groups, suggesting an EPO-mediated effect on heightening exploratory behavior ( $p < 0.05$ ). Furthermore, there were no differences between the time spent in the inner zone between the Phy+EPO and Psy+EPO groups when compared to the time spent in this zone by the sham group.

As expected, greater times spent in the outer zone, which suggests a

higher hesitancy to explore, were exhibited by the Phy and Psy groups compared with sham group, as reflected in Fig. 1D ( $p < 0.001$ ). However, when treated with EPO, both groups of stressed animals (Phy+EPO and Psy+EPO) exhibited smaller times in the outer zone which was significantly different from animals exposed to stress without treatment, suggesting an anti-anxiety effect of EPO ( $p < 0.05$ ). In addition, Phy+EPO and Psy+EPO groups did not exhibit significant differences in this parameter when compared with the time spent in the outer zone by the sham group. Time spent grooming and rearing were not found to significantly differ between groups.

The EPM was another behavioral test utilized to evaluate effects of EPO on stress affected cognitive behaviors. Lower degrees of anxiety or the effect of a treatment on reductions of anxiety are measured by relatively greater time expenditure in the open arms vs closed arms. Consistent with a greater level of anxiety following stress, Fig. 2A shows time spent in open arms was significantly smaller in Phy group ( $p < 0.01$ ) and Psy group ( $p < 0.05$ ) compared to the sham group. While the time spent in the open arms was higher in the two groups treated with EPO, this did not represent a difference when compared to the time spent by the stressed groups. However, unlike what was seen between the stress only groups, there were no differences in the EPO groups when compared to sham, suggesting an effect of EPO on reducing anxiety.

In addition, time spent in closed arms was affected by physical and psychological stress. As indicated in Fig. 2B, Phy and Psy groups spent more time in the closed arms as compared to the sham group ( $p < 0.05$ ), which was not a difference noted when this parameter was compared between the sham and EPO treated groups. The frequency ratio of entering open and closed arms was not significant among the groups (Fig. 2C).



**Fig. 1.** (A), Total distance moved decreased in groups that were exposed to physical (Phy) and psychological stress (Psy) in comparison with the sham group. (B), There was no significant difference in immobility duration time among groups. (C), Inner zone duration was lower in Phy and Psy groups, but EPO treatment was associated with a significantly greater duration in this parameter. (D), Outer zone duration was greater in Phy and Psy groups but treatment with EPO was associated with a significantly lower duration in this zone (\*:  $p < 0.05$ , \*\*\*:  $p < 0.001$  as compared to the sham group, ¥ and T:  $p < 0.05$  as compared to Phy and Psy groups; Erythropoietin= EPO).

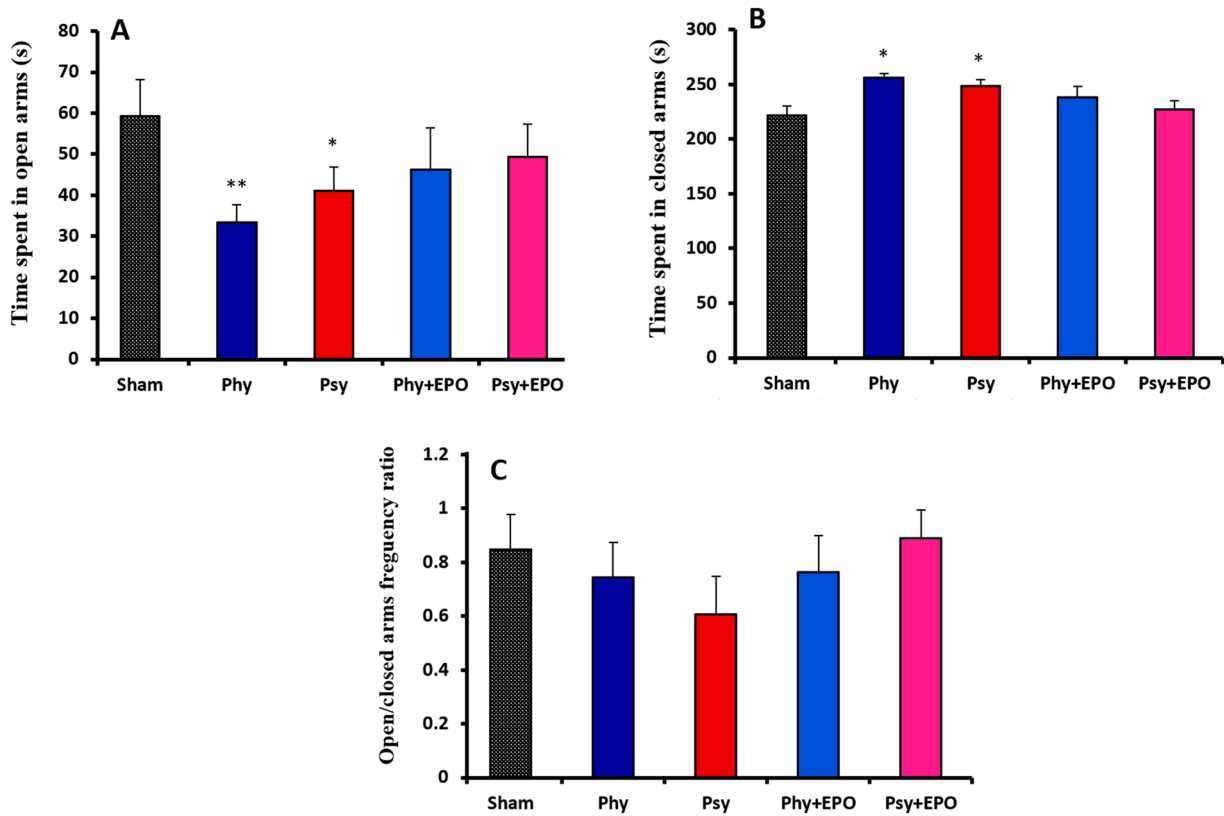


Fig. 2. (A) Time spent in the open arms was significantly lower in Phy and Psy groups as compared to the sham group. (B) Time spent in the closed arms was significantly greater in both Phy and Psy groups as compared to the time spend in closed arms by the sham group. (C) There wasn't significant difference among groups in the frequency ratio of open arm entries/total arm entries (\*:  $p < 0.05$ , \*\*:  $p < 0.01$ , as compared to the sham group).

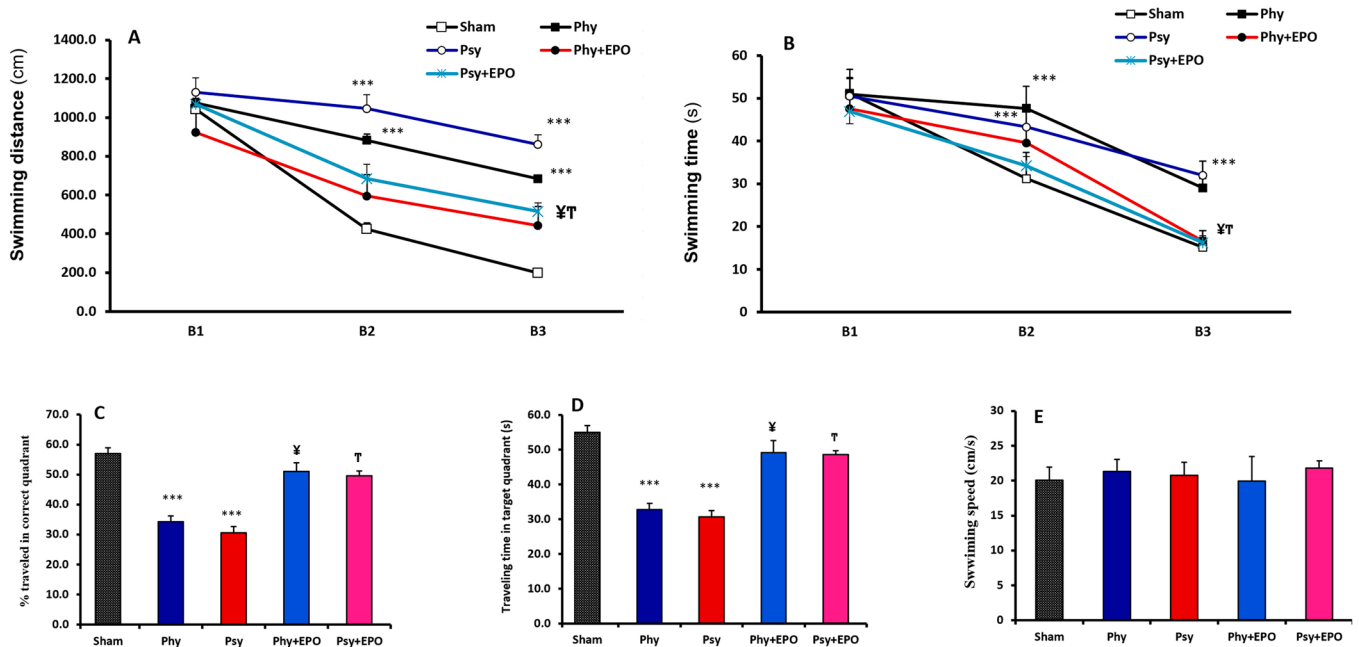


Fig. 3. The Phy and Psy groups swam a longer distance (A) and for a greater time (B) to find the platform in the MWM. Swimming distance and time were significantly lower in the Phy+EPO and Psy+EPO groups as compared to the groups that received only physical and psychological stress in the second and third blocks. (C, D) In the probe test, time to reach the correct quadrant was lower in both stressor groups compared to the sham, and EPO induced a reversal of this effect of stress. (E) No significant difference was observed in the swimming speed between the groups (\*\*\*:  $p < 0.001$  as compared to the sham group, ¥ and T:  $p < 0.05$  as compared to Phy and Psy groups; Erythropoietin= EPO).

### 3.2. The effect of stress and EPO administration on memory-related behaviors

The MWM was used to evaluate learning and memory in the different treatment groups. In Fig. 3A, traveled distance to reach platform showed a significant difference between groups. Swimming distance in the second and third blocks was significantly lower in Phy+EPO and Psy+EPO groups compared with groups that received only physical and psychological stress ( $p < 0.05$ ). Phy and Psy groups exhibited a significant greater swimming difference compared with the sham group ( $p < 0.001$ ); however, this difference was not seen when comparing this parameter between the sham group and the two stress groups treated with EPO. As indicated in Fig. 3B, Phy and Psy groups took a longer time to find the platform compared with sham ( $p < 0.001$ ) as well as the EPO treatment groups, suggesting the cognitive impact of stressors was ameliorated by EPO ( $p < 0.05$ ). In the probe test, the stress groups exhibited a smaller time and path of travel compared with the sham group ( $p < 0.001$ , Fig. 3C and D), which was a difference not noted when compared to these parameters in the EPO treated groups. Additionally, Phy+EPO and Psy +EPO groups showed significant differences when these two parameters were compared to the two groups that received only physical and psychological stress ( $p < 0.05$ ). No significant difference was seen in swimming speed between all of the groups.

## 4. Discussion

The main findings of the present study showed that EPO reduced adverse effects of psychological and physical stress on spatial memory, anxiety and locomotor activity. We assessed the effect of EPO against stress-induced motor and cognitive impairments and results demonstrated that EPO treatment could improve motor and cognitive dysfunctions induced by psychological and physical stress in rats. While results from other studies have suggested that EPO has neuroprotective effects in brain injury (Volpe, 2001; Rey et al., 2019), to our knowledge, this is the first report to demonstrate the neuroprotective effect of EPO against cognitive and motor dysfunctions induced by physical and psychological stress. When taken together with other studies, we conclude that EPO is a promising candidate for the treatment of neurological disorders involving neuronal damage due to physical or psychological stress.

Our results indicated that EPO administration decreased time spent in the inner zone and increased time spent in the outer zone when compared to the durations seen in these parameters in the physical and psychological stress groups. In the EPM, two types of stressors induced anxiety-like behaviors as indicated by the reduction of time spent in open arms and increased time spent in closed arms compared to sham animals, which did not differ from sham animals in stressed groups exposed to EPO, suggesting that these impairments were ameliorated by EPO. A protective effect of EPO against negative cognitive effects of stress was also observed in the MWM test. Results from this test showed that in the probe trial, EPO treatment resulted in a greater time in the correct quadrant, and decreased the swimming distance and time to reach the platform when compared to animals receiving stress alone. The results of this study are in line with other studies that showed anxiolytic and anti-depressant effects of EPO in animals (Dayyat et al., 2012; Osborn et al., 2013).

Effects on behaviors are likely due to neuroprotective actions of EPO (Ransome et al., 2007; Tahamtan et al., 2018). Consistent with this, EPO has been found to have strong anti-apoptotic, antioxidant, and anti-inflammatory properties in the brain including reducing both apoptotic and inflammatory factors, as well as activating neurotrophic and anti-oxidant signaling (Juul et al., 1999; Peng et al., 2020). In addition, other studies showed that EPO may increase expression of Brain-Derived Neurotrophic Factor (BDNF) or activate pathways such as phosphatidylinositol-3-kinase, Akt/protein kinase-B, and MAP kinases which are all cellular factors that could directly protect neurons from

damage (Byts et al., 2008). Moreover, an increase in BDNF after EPO administration may counterbalance the regulation of BDNF expression mediated by activation of the hypothalamic-pituitary-adrenal (HPA) axis, thereby limiting damage done to neurons through elevated glucocorticoids (Smith et al., 1995; Suliman et al., 2013). Further, other studies indicated that EPO regulates the secretion of adrenocorticotropic hormone (ACTH) and corticotropin-releasing hormone (CRH) by controlling the intracellular  $Ca^{2+}$  homeostasis in pituitary cells (Dey et al., 2015). EPO's regulation of HPA axis activation could prevent detrimental effects of HPA overactivation in pathological conditions on neurons and neural mechanisms important for memory (Tringali et al., 2007). Hippocampal CA3 neurons play a critical role in memory formation, and stress has been shown to reduce the density of spines in hippocampal CA3 cells, which has been associated with memory impairment. Blocking CRH in this region can reduce stress-induced damage (Chen et al., 2010). If EPO blocks CRH actions, this could result in a concurrent reduction in damage to spines in cognitive-related regions which could contribute to effects seen in animal and human studies in which the neuroprotective effects of EPO on cognitive function have been shown (Brines et al., 2000; Ehrenreich et al., 2007). Finally, stress significantly increases the stimulated production of pro-inflammatory cytokines, which are known to induce anxiety in animals (Maes et al., 1998). EPO, has been shown to decrease pro-inflammatory cytokines after traumatic brain injury in animals, suggesting EPO-mediated actions on reductions in cognitive-behaviors could be due to inhibition of the damaging effects of cytokines (de Miranda et al., 2011).

### 4.1. Limitation and future studies

Male rats were used in the current study to eliminate possibility of sex-based differences in stress responses. Further studies on female rats during their menstrual period and ovariectomized rats are warranted to evaluate the role of sex hormones on effects of EPO on stress-associated changes in behaviors. Another limitation in the current study is that we were unable to examine the mechanism by which EPO leads to reductions in negative effects of physical and psychological stress on locomotor and cognitive functions. Future studies should be conducted to examine at a molecular and cellular level the mechanisms by which EPO exerts a protective effect against the negative effects of stress on cognitive and locomotor behaviors.

In conclusion, we have demonstrated that physical and psychological stress leads to motor and cognitive dysfunctions and EPO effectively ameliorates against these adverse effects. Also, our findings showed that two different types of stressors had the same impact on the behaviors examined, and EPO was able to attenuate these dysfunctions. Further studies are required to explain the exact mechanisms of these alterations in order to develop more targeted EPO-based therapeutics.

### Conflict of interest

The authors declare no conflict of interest.

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### Author contribution

MF has conceived and designed the concept and road map of the study, searched the literature collected the data, and drafted the manuscript. MT designed the concept map, helped on data collection and reviewed the manuscript and figures. KAK and MS have critically reviewed the manuscript, designed the study, and helped in manuscript preparation. MS is the archival author and attests to the integrity of the

original data and the analysis reported in this manuscript. All authors have made substantive contribution and attest to approving the final manuscript.

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