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The protective effect of zeranol in cerebral ischemia reperfusion via p-CREB overexpression

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ABSTRACT

Aims: Cerebral ischemia reperfusion (I/R) is a neurovascular disease leading to cerebral damage. It was found that postmenopausal women are liable to more dangerous effects than men at same age in stroke. The objective of this study is to investigate the neuroprotective effect of zeranol against cerebral ischemia reperfusion in ovariectomized rats.

Main methods: 36 female wistar rats divided in to 3 groups: sham group, I/R group (where I/R was induced 7 weeks after ovariectomy), zeranol group (0.5 mg/kg every 3 days for 5 weeks before I/R). Cerebral ischemia reperfusion (I/R) was performed by bilateral common carotid artery occlusion then de-ligated to restore blood flow. After 24 h of reperfusion, rats performed cylinder test to evaluate behavioral dysfunction followed by decapitation. Brain tissues were collected for biochemical measures such as oxidative stress marker malondialdehyde, antioxidant markers reduced glutathione, inflammatory markers (interleukin-1 beta, tumor necrosis factor alpha, and inducible nitric oxide synthase), matrix metalloproteinase-9, adenosine triphosphate, brain derived neurotrophic factor, glucose transporter-3, phosphorylated c-AMP response element binding protein and finally nissl staining for histopathological examination.

Key findings: The zeranol administered group showed a reversal of neuronal damage caused by ischemia evidenced by the decrease in MDA, IL-1β, TNF-α, and MMP-9 levels, increase GSH, and ATP levels, decrease expression of iNOS in both regions cortex and hippocampus, increase protein level of p-CREB, GLUT-3 and BDNF, increase number of intact neuron cells in both regions and attenuated histological changes in both cortex and hippocampus regions.

Significance: Zeranol has neuroprotective potential against cerebral ischemia reperfusion in ovariectomized rats.

1. Introduction

Cerebral ischemia/reperfusion (I/R) injury causes cerebral damage through a complex cycle of pathophysiological events that occur through a short period of time. The brain is liable to energy depletion injuries and the damage was rely to oxidative stress as consequence of high rate of oxidative metabolic activity, relatively low antioxidant capacity and insufficient neuronal cell repair activity [1]. Ischemic stroke causes a reduction in blood flow which is enough to change normal cellular function. Reperfusion is key point for the remedy of ischemic stroke. However, the occurrence of post-reperfusion oxidation lesion is mainly due to large amounts of reactive oxygen species (ROS), that cause apoptosis, an inflammatory reaction, which accompanied with a blood brain barrier (BBB) disruption, and all finally leading to brain edema [2].

I/R injury leads to brain atrophy due to increased expression of nitric oxide, excitatory amino acids, cytokines, free radicals, mitochondrial respiratory enzymes damage, initiation programmed cell death, and microglia activation [3].

Cerebral ischemia causes a reduction of glucose which is the main energy source in the brain. Neuronal glucose uptake depends on glucose transporter isoform Glut3 at the plasma membrane [4].

Epidemiological studies revealed that stroke incidence increase with age and menopause women are more liable to stroke [5]. The occurrence of stroke in postmenopausal women is the same as in men. But, the undesirable effects (almost 60% deaths from stroke) have been

Abbreviations: ATP, adenosine triphosphate; BDNF, brain derived neurotrophic factor; CA1, cornu ammonis1; GLUT3, glucose transporter3; GSH, reduced glutathione; I/R, ischemia reperfusion; IL-1β, interleukin-1beta; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde (lipid peroxidation); MMP-9, matrix metalloproteinase-9; OVX, ovariectomized; p-CREB, phosphorylated cAMP response element binding protein; ROS, reactive oxygen species; TNF-α, tumor necrosis factor alpha; ZER, zeranol

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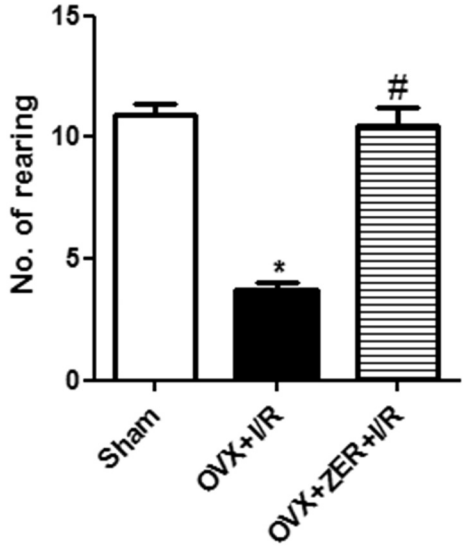

Fig. 1. Effect of zeranol on rearing number in cylinder test in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion.
Data were expressed as mean ± S.E.M, n = 12 in each group.
*Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$.
I/R: ischemia reperfusion, OVX: ovariectomized, ZER: zeranol.

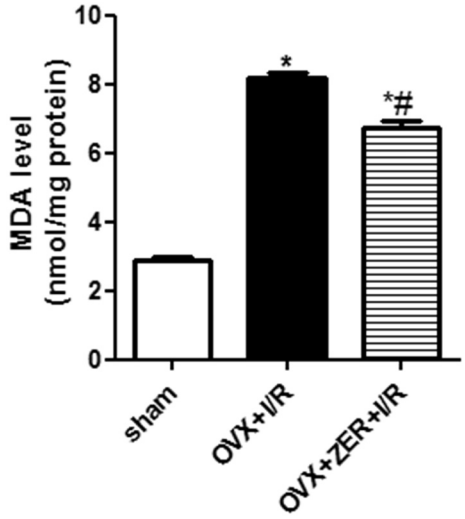

Fig. 2. Effect of zeranol on malondialdehyde (MDA) level in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion.
Data were expressed as mean ± S.E.M, n = 6 in each group
*Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$. I/R: ischemia/reperfusion, OVX: ovariectomized, ZER: zeranol.

recorded in the postmenopausal women, and as postmenopausal women are more threatened by stroke, it is a matter of urgent attention for this category [6].

Sex steroids are responsible for other functions than reproduction. The effects of sex steroids are involved in cognition, synaptic plasticity, memory and neurogenesis [5]. Previous studies have documented that ovariectomy causing behavioral, neurochemical and molecular defects associated with a decline in Brain-Derived Neurotrophic Factor (BDNF) and found that these defects can be partially returned to normal state by estradiol [5].

Reducing cerebral ischemic injury in experimental animals by estrogen replacement therapy has been well documented. It is therefore concluded that estrogen may also be efficient in inhibition of stroke in

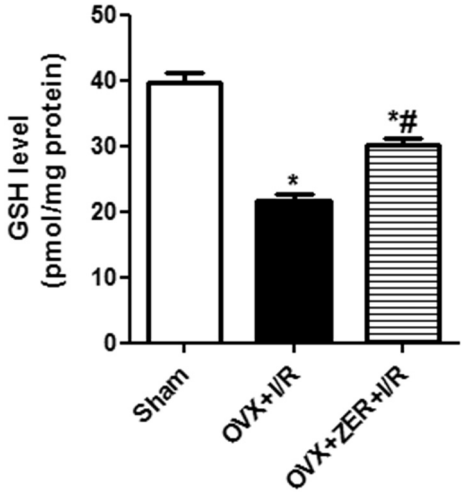

Fig. 3. Effect of zeranol on reduced glutathione level in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion.
Data were expressed as mean ± S.E.M, n = 6 in each group.
*Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$.I/R: ischemia/reperfusion, OVX: ovariectomized, ZER: zeranol.

Table 1
Effect of zeranol on level of IL-β and TNF-α in rat brain tissue after I/R injury.

Parameters	Groups		
	Sham	OVX + I/R	OVX + ZER + I/R
TNF-α level (Pg/mg protein)	33.22 ± 0.86	55.14 ± 1.30*	45.53 ± 0.79*#
IL-1β level (Pg/mg protein)	77.93 ± 1.94	143.10 ± 3.27*	117.60 ± 2.97*#

Data represented as mean ± S.E.M, n = 6 rat in each group.
* Significantly different from sham group at $p \leq 0.05$.
# Significantly different from I/R group at $p \leq 0.05$. I/R: ischemia/reperfusion; OVX: ovariectomized; ZER: zeranol.

postmenopausal women but the adverse effects associated with estrogen a search for substitutes with less side effects but a same protective effect such as selective estrogen receptor modulators and phytoestrogen [6].

Plant-derived phytoestrogens act as potential substituent for estrogen. The phytoestrogen α-Zearalanol (zeranol), associated with low side-effects on the reproductive system in relation with animal-derived estrogen, is found to protect against cell injury with low side-effects on uterus and breast [7,8].

Zeranol is a reduced product of Gibberella zeae metabolites that is found in plants and vegetables, soybeans, wheat, grapes, radishes, celery, spinach, and apples, and able to metabolize safely in the body. Previous reports reported that zeranol has ability to conserve oxidant-antioxidant balance. This role thereby gives zeranol potential to prevent the neuron damage due to oxidative stress [8].

2. Materials and methods

2.1. Chemicals

Zeranol was purchased from Sigma Aldrich (St Louis, MO, USA). Rabbit polyclonal anti-phosphorylated c-AMP response element binding protein (p-CREB, 1:1000) and inducible nitric oxide synthase (iNOS, ready to use) were purchased from Thermo Fisher Scientific, Waltham, CA, USA. MDA colorimetric kit was purchased from BioVision Incorporated, USA. Reduced glutathione (GSH) colorimetric kit was

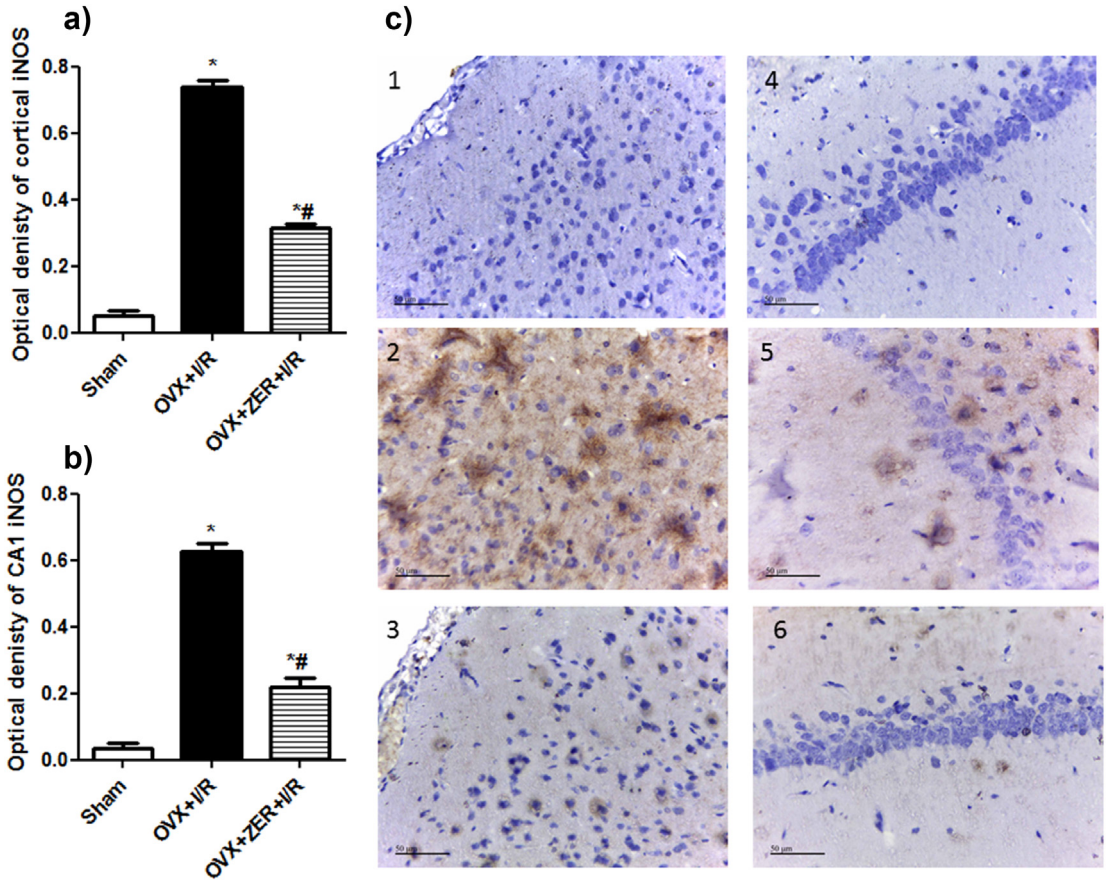

Fig. 4. a: Effect of zeranol on cortical inducible nitric oxide synthase (iNOS) in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion. Data were expressed as mean ± S.E.M, n = 6 in each group.
*Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$. I/R: ischemia/reperfusion; OVX: ovariectomized; ZER: zeranol.
b: Effect of zeranol on hippocampal inducible nitric oxide synthase (iNOS) in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion. Data were expressed as mean ± S.E.M, n = 6 in each group.
*Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$. I/R: ischemia/reperfusion; OVX: ovariectomy; ZER: zeranol.
c: Representative photomicrographs showing increase in expression of iNOS in I/R group in both regions cortex and hippocampus (CA1) (2,5) as compared to sham group (1.4), while zeranol group showed decrease in expression of iNOS in both regions (3,6) as compared to I/R group (×40), scale bar = 50 μm. I/R: ischemia/reperfusion; OVX: ovariectomized; ZER: zeranol.

purchased from Enzo Life Sciences, Switzerland. Interleukin-1 beta (IL-1β) ELISA kit was purchased from Cohesion Biosciences, UK. Tumor necrosis factor alpha (TNF-α), matrix metalloproteinase-9 (MMP-9) ELISA kit were purchased from CUSAIBO, USA. Adenosine triphosphate (ATP) ELISA kit was purchased from Blue Gene Biotech. for life science, China. Glucose transporter-3 (GLUT-3) and brain derived neurotrophic factor (BDNF) ELISA kit were purchased from Cloud-Clone Corp., USA. All other chemicals were of the highest available commercial grade.

2.2. Animal

Thirty six female adult wistar rats were included in this study, weighing 180–200 g. The rats were supplied from the breeding unit of the Egyptian Organization of Biological products and Vaccines (Helwan, Egypt). Animals had free access to standard pellet diet and water all through the day and housed with normal light dark cycles for 1 week before experiment at controlled temperature (22 °C + 2 °C).

Animal handling and experimental protocol were approved by the research ethics, animal care and use committee, Faculty of Pharmacy, Helwan University (Protocol Number: 003A-2018).

2.2.1. Surgical procedure

2.2.1.1. Ovariectomy operation. Animals were anesthetized with chloral hydrate (360 mg/kg i.p.) and the surgical site was shaved and cleansed thoroughly with Betadine. A midline abdominal incision was made, and the junction between the ovaries and uterus were located and tied with 2.0 silk. Ovaries were removed bilaterally [9]. Vaginal smears were obtained to confirm cessation of oestrous cycling in OVX animals.

2.2.1.2. Induction of cerebral ischemia/reperfusion (I/R). Overnight-fasted rats were anesthetized with chloral hydrate (360 mg/kg, i.p.) at the time of the operation. Rats were subjected to ischemia, where the right and left common carotid arteries were exposed by a midline ventral incision in the neck. The bilateral carotid artery were separated from the adjacent tissues and vagus nerve. Ischemia was induced by bilateral clamping (ligation) of the common carotid arteries for 10 min. Following cerebral ischemia, the arteries were de-clamped (de-ligated) to restore circulation. The skin was sutured with waxed silk stitches. Reperfusion was allowed for 24 h. Rats of the sham group were exposed to the same procedure except for carotid occlusion. Rectal temperature was maintained at 37 ± 0.5 °C throughout the experiment to prevent cerebral hypothermia [10].

2.3. Experimental design

The experiment dealt with the determination of the protective effect of zeranol treatment on the cerebral ischemic reperfusion injury of the ovariectomized rats. The rats were randomly divided into three

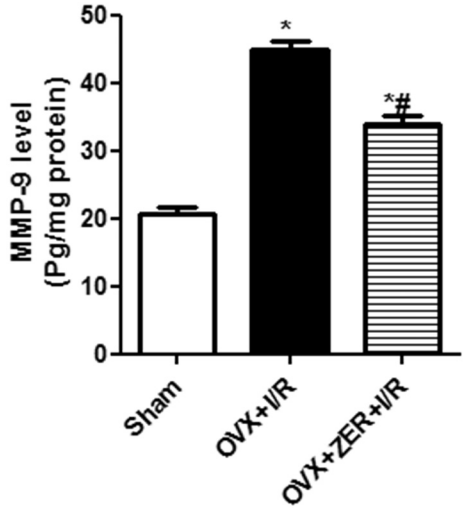

Fig. 5. Effect of zeranol on matrix metalloproteinase (MMP-9) level in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion. Data were expressed as mean ± S.E.M, n = 6 in each group. *Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$. I/R: ischemia/reperfusion; OVX: ovariectomized; ZER: zeranol.

Table 2
Effect of zeranol on level of ATP and GLUT-3 in rat brain tissue after I/R injury.

Parameters	Groups		
	Sham	OVX + I/R	OVX + ZER + I/R
ATP level (ng/mg protein)	23.20 ± 0.97	9.81 ± 0.57*	14.58 ± 0.35*#
GLUT-3 level (ng/mg protein)	7.02 ± 0.30	3.15 ± 0.16*	4.14 ± 0.11*#

Data represented as mean ± S.M.E, n = 6 rat in each group.
* Significantly different from sham group at $p \leq 0.05$.
# Significantly different from I/R group at $p \leq 0.05$. I/R: ischemia/reperfusion; OVX: ovariectomized; ZER: zeranol.

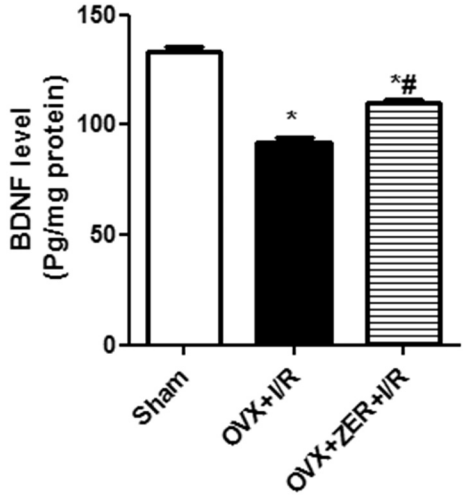

Fig. 6. Effect of zeranol on brain derived neurotrophic factor (BDNF) level in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion. Data were expressed as mean ± S.E.M, n = 6 in each group. *Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$. I/R: ischemia/reperfusion; OVX: ovariectomized; ZER: zeranol.

groups, in each group = 12.

Group 1 (Sham group): neither exposed to ovariectomy nor to ischemia, rats received vehicle of zeranol for 5 weeks.

Group 2 (OVX + I/R) Ovariectomized rats that weren't treated, only received vehicle of zeranol for 5 weeks and underwent cerebral I/R injury 7 weeks after ovariectomization.

Group 3 (OVX + ZER + I/R) Ovariectomized rats treated 2 weeks after ovariectomization with zeranol (0.5 mg/kg/olive oil; i.p. [11]). Treatment continued for 5 weeks every 3 days followed by cerebral I/R injury.

2.4. Parameters

2.4.1. Rearing behavior in cylinder test

When placed in a clear cylinder, rats will engage in exploratory behavior, including rearing. During rearing behavior, the forelimbs will contact the wall of the cylinder. For this test, the rat will be placed in a clear plexiglass cylinder (diameter = 20 cm, height = 30 cm) for 5 min. The number of rears will be counted [12].

2.4.2. Biochemical parameters

2.4.2.1. Tissue sampling and preparation. After behavioral experiment, rats were sacrificed by decapitation and brains were removed then rinsed with ice cold phosphate buffer and divided into two set, the first set in which 6 rat brains tissues were stored at −80 °C for biochemical studies such as reduced glutathione (GSH), malondialdehyde (MDA), interleukin-1beta (IL-1β), tumor necrosis factor alpha (TNF-α), matrix metalloproteinase-9 (MMP-9), glucose transporter-3 (GLUT-3), and adenosine triphosphate (ATP) which were homogenized in phosphate buffer except GSH in 1× assay buffer to obtain 10% for all except 5% for GLUT-3 and BDNF according to kit instruction. While the other set (6 rat brains) were divided for two hemispheres, the right ones were stored at −80 °C, which were used for western blot assay for measuring level of p-CREB and the left hemispheres were used for immunohistochemistry staining (iNOS), toluidine blue staining and finally histopathology in both regions cortex and hippocampus (CA1).

2.4.2.2. Determination of MDA, GSH, IL-1β, TNF-α, MMP-9, GLUT-3, ATP and BDNF in brain homogenate. The brain tissue level of MDA, GSH, IL-1β, TNF-α, MMP-9, GLUT-3, ATP, and BDNF were determined using commercial available kit, according to kit manufacturer instruction. All parameters were measured in the supernatant of brain homogenate except GSH. MDA level was determined with the thiobarbituric acid method by using method of [13], while GSH was determined with Ellman's reagent by using method of [14]. The remaining parameters were measured by enzyme-linked immunosorbent assay (ELISA) kit. The samples were analyzed with a spectrophotometer.

2.4.3. Protein estimation

Protein was assessed by the method of lowery [15] using bovine serum albumin as standard.

2.4.4. Histopathology

At the end of the reperfusion period (after cylinder test), 6 left hemispheres from each group were removed and fixed in 10% neutral-buffered formalin for 24 h. Each left hemisphere was embedded in paraffin blocks. Sagittal sections of six μm thickness were obtained (containing partial cortex which was examined with special focus on (outer molecular layer, outer granular layer and outer pyramidal layer) where most of lesions were recorded and hippocampus) then stained with 1% toluidine blue at 50^0c for 30 min to count intact neuron cells which showing cellular details with intact lightly stained nuclei, while damaged neurons are darkly stained having no cellular details and without clear bounders between nuclear and cytoplasmic compartment as Lan et al [16] in five fields per slide and the average was calculated. In addition to hematoxylin and eosin (H&E) stain for histopathological

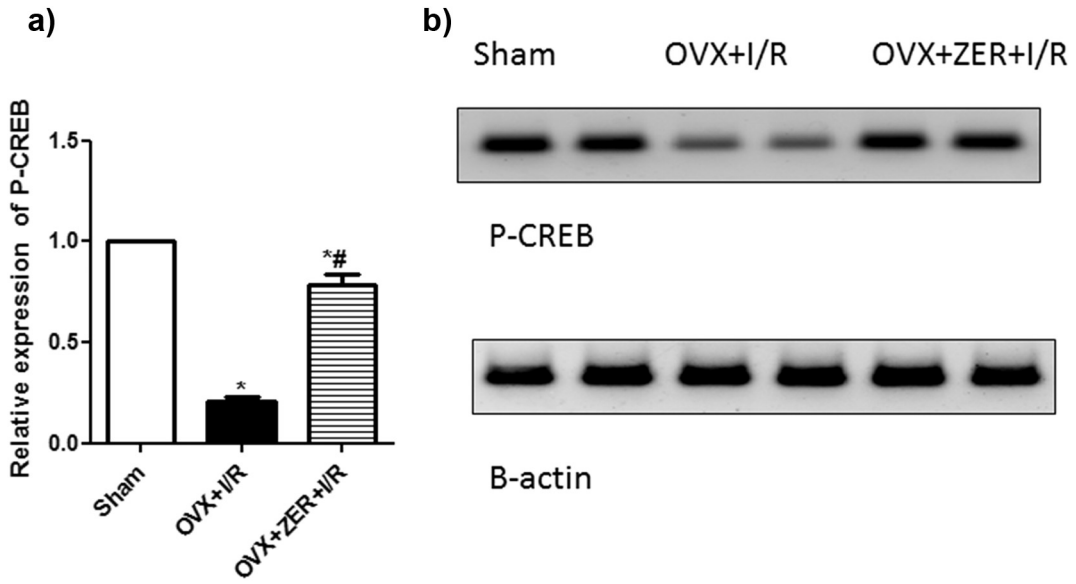

Fig. 7. (a) Effect of zeranol on phosphorylated c-AMP response element binding protein (p-CREB) in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion.
Data were expressed as mean ± S.E.M, n = 6 in each group.
*Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$; (7b) Representative Images of western blotting of p-CREB in brain tissue after 10 min ischemia followed by 24 h reperfusion showed increased expression of p-CREB in ZER group. I/R: ischemia/reperfusion; OVX: ovariectomized; ZER: zeranol.

examination according to the method of [17]. The sections were examined for the histopathological findings of any changes.

2.4.5. Immunohistochemistry

The technique used for immunostaining was the Labeled Streptavidin Biotin (LSAB) method according to [18]. 5 µm thick paraffin embedded tissue section were prepared. After deparaffinization sections were immersed in 3% H2O2 in methanol for 10 min to block endogenous peroxidase. Nonspecific binding sites were blocked by incubating the sections in 10% goat serum for 10 min to suppress nonspecific binding followed by incubation with iNOS Rabbit polyclonal antibody - ready to use (Thermo Fisher Scientific, Waltham, CA, USA) at room temperature for 60 min, followed by incubation with biotinylated goat anti-rabbit IgG at room temperature for 10 min. After 3 times of 3 min PBS rinses sections were incubated for 10 min with streptavidin horseradish peroxidase by using Histostatin-Bulk-SP IHC, broad spectrum (Thermo Fisher Scientific, Waltham, CA, USA). The antibody binding sites were visualized by incubation with diaminobenzidine (DAB) for 10 min. After that Washing by PBS then counter staining with hematoxylin, dehydrated and clearing in xylene then cover slipping for microscopic examination.

Finally, all slides were photographed under a light microscope (magnification, ×400; Olympus BX50), the intensity of iNOS in the cortex and hippocampus (CA1) in each section was evaluated using computerized image analysis Leica Qwin 500 (LEICA, Image system Ltd., Cambridge, UK) which consist of a leica DM-LB microscope with JVC color video camera attached to a computer system Leica Q 500 IW. Five random fields per slide were analyzed and the average was calculated, and expressed the integrated optical density.

2.4.6. Western blot

Brain tissue was homogenized in cell RIPA lysis buffer (Bio Basic Inc., Canada), followed by centrifugation at 4 °C (16,000 × *g* for 30 min) to collect the supernatant. Protein concentration was determined by Bradfort protein assay kit (Bio Basic Inc., Canada). 20 µg protein concentration of each sample was loaded with an equal volume of 2× Laemmli sample buffer (Bio-Rad Laboratories Inc., USA) and the mixture was boiled at 95 °C for 5 min to ensure denaturation of protein before loading on polyacrylamide gel electrophoresis (SDS-PAGE), followed by electro-transfer to polyvinylidene difluoride (PVDF) membranes. After blocking with Tris-buffered saline with Tween (TBST) buffer containing 3% bovine serum albumin (BSA) at room temperature for 1 h, membranes were incubated with rabbit p-CREB primary polyclonal antibodies (1:1000) overnight at 4 °C. After washing three times with 0.05% TBST, the membranes were incubated with horseradish peroxidase (HRP) goat anti-rabbit secondary polyclonal antibody (1:1000; Novus Biological, Littleton Co, USA) at room temperature for 1 h under vibration. An enhanced chemilumescent (ECL) detection system (Bio-Rad, Laboratories, Inc., Hercules, CA, USA) was used to detect the signals using the chemiluminescent substrate (Clarity Western ECL substrate – Bio-Rad, Hercules, CA, USA). The chemiluminescent signals were captured using a CCD camera-based imager. Image analysis software was used to read the band intensity of the target proteins against control sample after normalization by beta actin on the Chemi Doc MP imager.

2.5. Statistical analysis

Data were expressed as mean ± standard error of mean. Results were analyzed statistically using one way ANOVA followed by **Tukey-Kramer** [19] as post hoc test. Statistical analysis and graphical representations were performed using GraphPad Prism, version 5 (GraphPad software, Inc., San Diego, CA, USA). A p-value of 0.05 or less was considered as statistically significant.

3. Results

3.1. Effect of zeranol treatment on rearing number in cylinder test after I/R injury

Cerebral ischemia for 10 min followed by reperfusion for 24 h in ovariectomized rats showed significant decrease in exploratory behavior by reducing the number of rearing in cylinder test by approximately 66%when compared with sham group, while pretreatment with zeranol showed significant increase in the number of rearing by approximately 180% when compared with I/R group (Fig. 1).

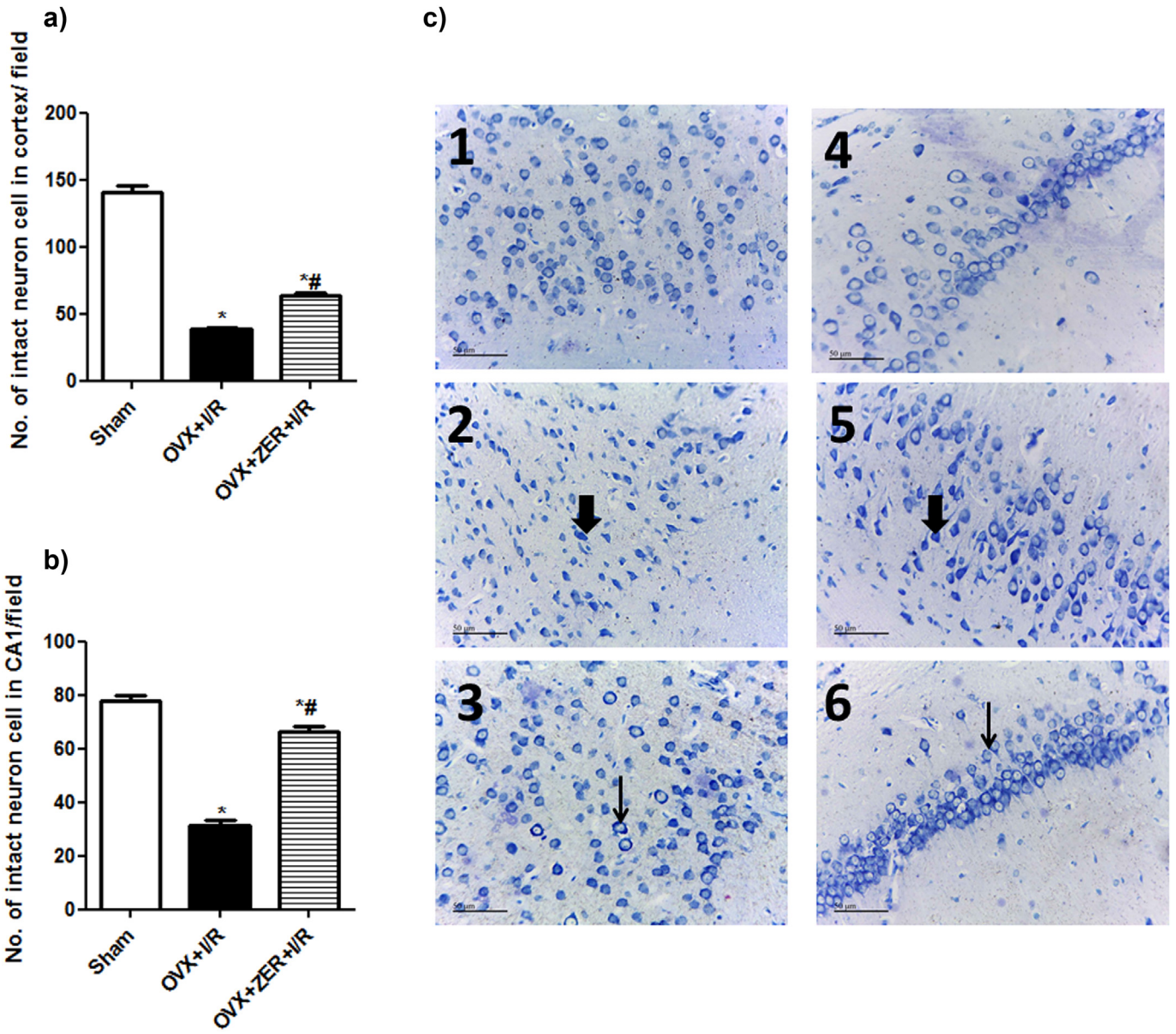

Fig. 8. a: Effect of zeranol on number of intact cortical neuron cell per field in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion. Data were expressed as mean ± S.E.M, n = 6 in each group.
*Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$. I/R: ischemia/reperfusion; OVX: ovariectomized; ZER: zeranol.
b: Effect of zeranol on number of intact neuron cell in CA1 per field in ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion. Data were expressed as mean ± S.M.E, n = 6 in each group.
*Significantly different from sham group, #significantly different from I/R group at $p \leq 0.05$. I/R: ischemia/reperfusion; OVX: ovariectomized; ZER: zeranol.
c: Representative photomicrographs of nissl staining of the cortex (1, 2, 3) and hippocampus (CA1) region (4, 5, 6) showed that in I/R group (2, 5) neuron cell appeared shrunken, had deep staining (thick arrow) and decreased number of intact neuron cells in both regions as compared to sham group (1, 4) while pre-treatment with zeranol showed increase in number of intact neuron cells in both regions (fine arrow). In which 1, 4 refers to sham group, 2, 5 refers to I/R group and 3, 6 refers to zeranol group (×40), scale bar = 50 μm.

3.2. Effect of zeranol treatment on oxidative stress marker (MDA) and antioxidant marker (GSH) in brain tissue after I/R injury

In ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion, the level of brain MDA as a marker of lipid peroxidation was significantly raised by 180.7% as compared to the sham-operated rats. In zeranol treated group, the level of MDA was significantly attenuated by 17.5% as compared to I/R group (Fig. 2). Concerning GSH level, our results revealed that the GSH level in the brain tissues of I/R group was significantly depleted by 45.6% as compared to sham-operated group. The depleted level of GSH was significantly restored in zeranol-treated group by 40.4% as compared to I/R group (Fig. 3).

3.3. Effect of zeranol treatment on inflammatory markers (IL-1β, TNF-α) in rat brain tissue after I/R injury

As shown in Table 1, the level of IL-1β and TNF-α in the brain tissues of I/R group were significantly elevated by 85% and 65%, respectively, compared to sham-operated animals. However, zeranol treated group reduced the level of IL-1β and TNF-α, by 17.8% and 17.4%, respectively, compared to I/R group.

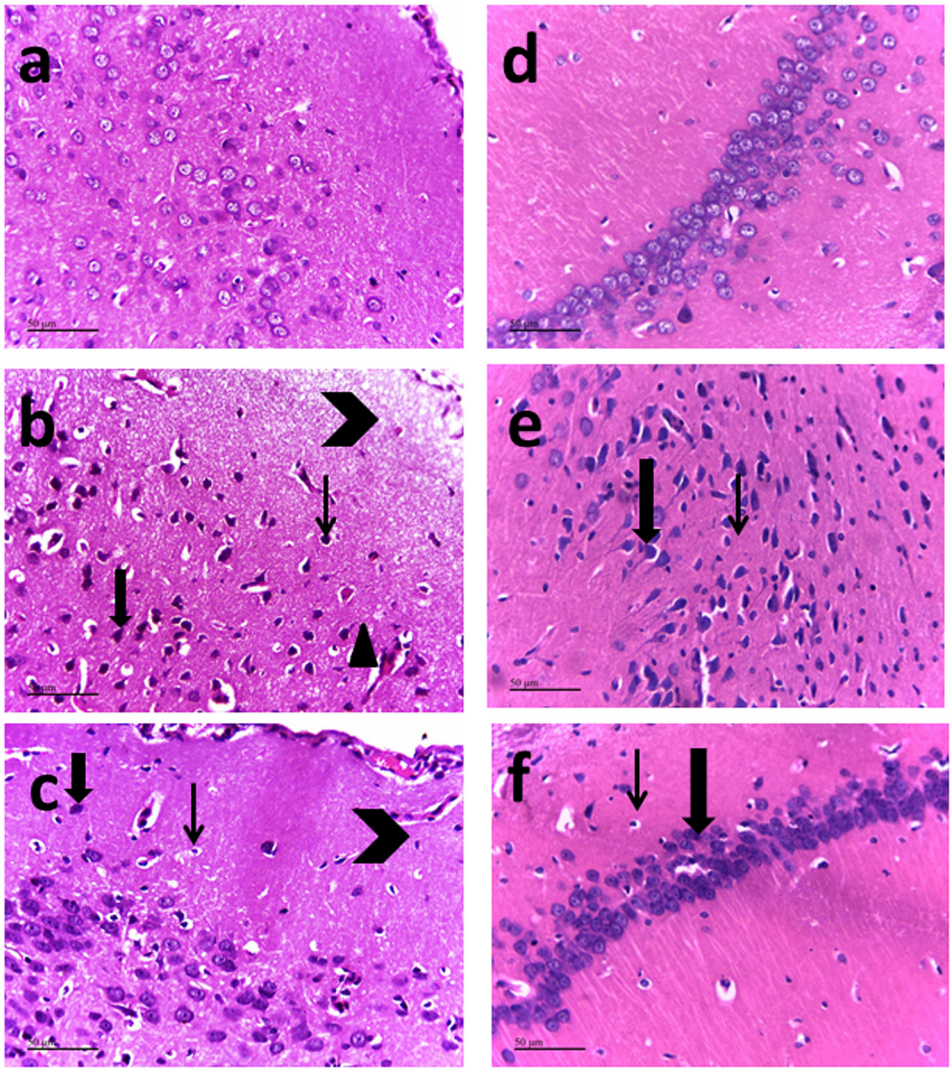

Fig. 9. Representative photomicrographs of brain section in both regions cortex (a: sham group, b: I/R group, c: zeranol group) and hippocampus (CA1) (d: sham group, e: I/R group, f: zeranol group) showed effect of zeranol on histopathological changes in ovariectomized rats exposed to 10 min cerebral ischemia followed by 24 h reperfusion (H&EX40), scale bar = 50 μm.

3.4. Effect of zeranol on iNOS expression determined by immunohistochemistry in both regions cortex and hippocampus after I/R injury

In ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion, the optical density of positive iNOS cells was significantly increased in both cortex and hippocampus CA1 regions by 1376 and1694% respectively as compared to the sham group. In zeranol treated group, the optical density of positive iNOS cells was decreased significantly by 56.6 and 65%respectively as compared to I/R group (Fig. 4a, b, c).

3.5. Effect of zeranol on blood membrane barrier integrity by measuring MMP-9 in rat brain tissue after I/R injury

In ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion, the level of brain MMP-9 was significantly elevated by 117.4% as compared to the sham-operated rats. In zeranol treated group, the level of MMP-9 was significantly attenuated by 24.15% as compared to I/R group (Fig. 5).

3.6. Effect of zeranol on energy level (ATP, GLUT-3) in rat brain tissue after I/R injury

As shown in Table 2, the level of ATP and GLUT-3 in the brain tissues of I/R group were significantly decreased by 57.7% and 55.13%, respectively, compared to sham-operated animals. However, zeranol treated group increased the level of ATP and GLUT-3, by 48.6% and 31.43%, respectively, compared to I/R group.

3.7. Effect of zeranol on neurogenesis and neuroplasticity (BDNF) in rat brain tissue after I/R injury

In ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion, the level of brain BDNF was significantly decreased by 30.81% as compared to the sham-operated rats. In zeranol treated group, the level of BDNF was significantly increased by 19.4% as compared to I/R group (Fig. 6).

3.8. Effect of zeranol on phosphorylated c-AMP response element binding protein (p-CREB) in rat brain tissue after I/R injury

In ovariectomized rats subjected to 10 min ischemia followed by 24 h reperfusion, the level of brain p-CREB was significantly decreased

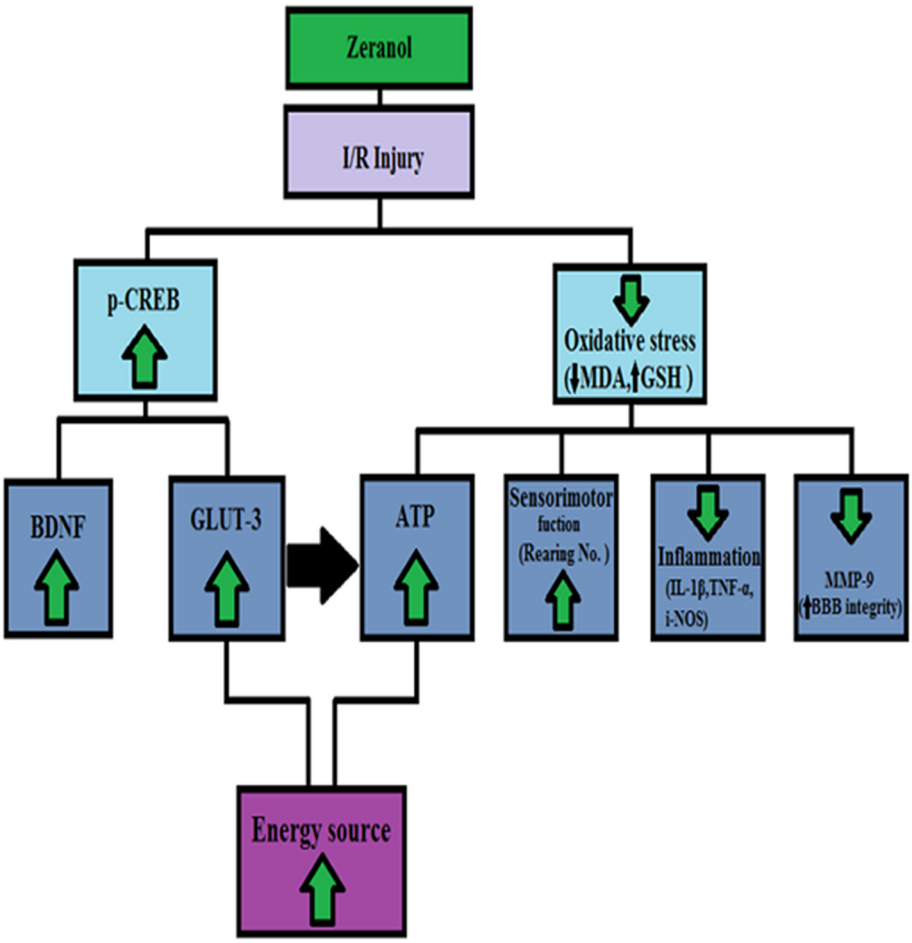

Fig. 10. schematic representative the mechanism of action of zeranol in I/R injury in ovariectomized rats.
p-CREB (phosphorylated c-AMP response element binding protein); GLUT-3 (glucose transporter-3); ATP (adenosine triphosphate); IL-1β (interleukin-1 beta); TNF-α (tumor necrosis factor-alpha); iNOS (inducible nitric oxide synthase); MMP-9 (matrix metalloproteinase-9); BBB (blood brain barrier); MDA (malondialdehyde); GSH (reduced glutathione).

by 80%as compared to the sham-operated rats. In zeranol treated groups, the level of p-CREB was significantly increased by 283% as compared to I/R group (Fig. 7a, b).

3.9. Effects of zeranol on neuronal injury in rat brain tissue after I/R injury

Nissl staining showed neuronal injury in cortex and hippocampus (CA1) region at 24 h after reperfusion. The images showed that most cells were shrunk in the ischemic cortex, hippocampus (CA1) region and had deep color staining indicative of injury in I/R group. However, these characteristic changes were not observed in the sham group and were improved by zeranol treatment. The intact neuron cells number of the cortex and the hippocampus region were smaller in I/R group than those of sham operated group (38.33 ± 1.02 vs. 140.8 ± 4.56, $p < 0.05$ for cortex; 31.50 ± 1.82 vs. 73 ± 1.79, $p < 0.05$ for the hippocampus, respectively). While pretreatment with zeranol showed increase in intact neuron cells number as compared to I/R group in both regions (63.33 ± 2.53, 66.33 ± 1.84 respectively) (Fig. 8a, b, c).

3.10. Effect of zeranol on histopathological changes in both regions cortex and hippocampus after I/R injury

Brain section of sham group showed normal histological structure in both regions cortex and hippocampus (Fig. 9a, d).

Brain section of ovariectomized I/R group showed many shrunken neuron cells with dark basophilic nucleus (thick arrow), many glia cell infiltration (fine arrow), congestion in blood vessels (triangle) and edema (arrow head) in cerebral cortex (Fig. 9b) while, in hippocampus (CA1) region showed disarrangement of pyramidal cells, many shrunken irregularly shaped neurons with dark basophilic nuclei (thick arrow) and many glia cells (fine arrow) (Fig. 9e).

However, intraperitoneal administration of zeranol prior to I/R showed improvement in the histological properties, fewer numbers of shrunken dark basophilic neuron cells (thick arrow), mild glia cell infiltration (thin arrow) and mild edema (arrow head) in cerebral cortex (Fig. 9c), while CA1 region showed more organized apparent intact pyramidal cells, few scattered shrunken neurons with dark basophilic nuclei (thick arrow) and mild infiltration of glia cells (thin arrow) (Fig. 9f).

4. Discussion

Functional defects following ischemic stroke are often notable and therefore it is importance to examine changes in animal attitude after cerebral ischemia [20]. In the current study, sensorimotor function was investigated by the cylinder test.

Pretreatment with zeranol decreased sensorimotor dysfunction after I/R injury. This can be due to the ability of zeranol to maintain oxidant-antioxidant balance [8]. Additionally, our results show that zeranol administration alleviated the oxidative stress caused by I/R injury as evidenced by the decrease in MDA level and increase in GSH level.

Furthermore, an imbalance between oxidant and antioxidant

markers in I/R injury is a precursor to an increase in inflammation seen by an increase in TNF-α, iNOS, and IL-1β [21,22]. We obtained similar results in our I/R injury model. This may be due to the up-regulation of NF-κB. We also find that zeranol administration prior to I/R injury caused a decrease in inflammation as evidenced by the decrease in TNF-α, iNOS, and IL-1β.

Oxidative stress and the subsequent production of reactive oxygen species are also responsible for the damage to the blood brain barrier in I/R injury as shown by [23]. Our experimental model shows that MMP-9 level is increased which suggests blood brain barrier damage. Zeranol was able to significantly alleviate this damage.

In I/R injury, the lack of oxygen and the resulting oxidative stress caused a fall in ATP level which is similar to the finding of [24,25]. However, the animals administered zeranol have increased levels of ATP after I/R injury. This may be due to the deactivation of F0F1ATPase activity leading to an increase in ATP level. This has been previously shown in the studies performed by [26,27,28] but with different estrogens. The increase in ATP level with zeranol may also be linked to GSH level increase similar to the results found by [29,30].

Neuronal survival and plasticity were assessed by measuring expression of p-CREB and the level of its downstream factor BDNF [31,32]. We found that zeranol pretreatment caused an increase in the expression of p-CREB accompanied with an increase in BDNF level. These results are similar to those found by [33,34,35] in different I/R models. Therefore, neuronal survival was improved after I/R injury by zeranol and this is similar with [36].

Phosphorylated CREB is also essential for the transcription of GLUT3 gene [37], while estrogen deprivation causes a decrease in the expression of GLUT3 [38].

Our results show that p-CREB upregulation by zeranol administration caused an increase in the expression of GLUT3 which is the main glucose transporter in neurons. Therefore, glucose is available in the neurons for the cell's metabolism and survival.

The histopathological examination supports these findings by showing an increase in intact neurons.

On the other hand, increase of GLUT3 expression and the availability of glucose inside the neuron give rise to the ATP level [38]. This suggests that ATP levels increase after zeranol administration and I/R injury via two mechanisms: the decrease in oxidative stress and the availability of glucose inside the cells.

The mechanism of action of zeranol in I/R injury in ovariectomized rats is illustrated in Fig. 10.

5. Conclusion

We conclude that the phytoestrogen zeranol improves I/R outcome in ovariectomized rats by righting the oxidant-antioxidant balance with subsequent decrease in inflammation and sensorimotor dysfunction. But neuronal survival was maintained by upregulation of p-CREB and GLUT3. And the increase in available glucose and ATP levels provide the neuron with its two essential energy sources for survival.

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Conflict of interest

The authors declare that there are no conflicts of interest.

References

[1] G.H. Heeba, A.A. El-Hanafy, Nebivolol regulates e-NOS and i-NOS expression and alleviates oxidative stress in cerebral ischemia/reperfusion injury in rats, Life Sci. 90 (11 – 12) (2012) 388–395.
[2] P. Wicha, J. Tocharus, A. Janyou, J. Jittiwat, C. Changtam, A. Suksamrarn, C. Tocharus, Hexahydrocurcumin protects against cerebral ischemia/reperfusion injury, attenuates inflammation, and improves antioxidant defenses in a rat stroke model, PLoS ONE 12 (12) (2017) e0189211.
[3] Y. Yao, L. Chen, J. Xiao, C. Wang, W. Jiang, R. Zhang, J. Hao, Chrysin protects against focal cerebral ischemia/reperfusion injury in mice through attenuation of oxidative stress and inflammation, Int. J. Mol. Sci. 15 (2014) 20913–20926.
[4] C.Y. Huang, Y.F. Liou, S.Y. Chung, P.Y. Pai, C.B. Kan, C.H. Kuo, C.H. Tsai, F.F. Tasi, J.J. Chen, J.Y. Lin, Increased expression of glucose transporter3 in gerbil brains following magnesium sulfate treatment and focal cerebral ischemia injury, Cell Biochem. Funct. 28 (2010) 313–320.
[5] N. Younan, S. El-Attar, D. Sabry, Progesterone as a neuroprotective treatment in brain ischemic injury in ovariectomized rats: relation to brain derived neurotrophic factor, synaptic plasticity and astrocytes, Medical Journal of Cairo University 80 (2) (2012) 67–80.
[6] S. Wang, H. Wei, M. Cai, Y. Lu, W. Hou, Q. Yang, H. Dong, L. Xiong, Genistein attenuates brain damage induced by transient cerebral ischemia through up-regulation of ERK activity in ovariectomized mice, Int. J. Biol. Sci. 10 (4) (2014) 457–465.
[7] Y. Dong, N. Yang, Y. Liu, Q. Li, P. Zuo, The neuroprotective effects of phytoestrogen α-zearalanol on β-amyloid-induced toxicity in differentiated PC-12 cells, Eur. J. Pharmacol. 670 (2–3) (2011) 392–398.
[8] Y. Dong, Y. Wang, Y. Liu, N. Yang, P. Zuo, Phytoestrogen α-zearalanol ameliorates memory impairment and neuronal DNA oxidation in ovariectomized mice, Clinics (Sao Paulo) 68 (9) (2013) 1255–1262.
[9] K.D. Prongay, A.D. Lewis, P.D. Hurn, S.J. Murphy, Dietary soy may not confound acute experimental stroke infarct volume outcomes in ovariectomized female rats, Lab. Anim. 44 (3) (2010) 238–246.
[10] S. Ozbilgin, O. Yılmaz, B.U. Ergur, V. Hancı, S. Ozbal, S. Yurtlu, S.F. Gunenc, B. Kuvaki, B.A. Kucuk, A.R. Sisman, Effectiveness of sugammadex for cerebral ischemia/reperfusion injury, The Koahsiung Journal of Medical Sciences 32 (6) (2016) 292–301.
[11] Y.L. Dong, Y. Yue, F.H. Liu, S.Y. Lang, X.C. Zhang, S.L. Dai, Q.S. Ge, P.P. Zuo, Treatment with phytoestrogen α-zearalanol might protect neurons of hippocampus in ovariectomized rats, Endocrine 30 (3) (2006) 249–254.
[12] J.P. Cannon, V. Tapias, H.M. Na, A.S. Honick, R.E. Drolet, J.T. Greenamyre, A highly reproducible rotenone model of Parkinson's disease, Neurobiol. Dis. 34 (2009) 279–290.
[13] M. Mihara, M. Uchiyama, Determination of malonaldhyde precursor in tissues by thiobarbituric acid test, Anal. Biochem. 86 (1) (1978) 271–278.
[14] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1) (1959) 70–71.
[15] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with Folin phenol reagent, J. Biol. Chem. 193 (1) (1951) 265–275.
[16] R. Lan, J. Xiang, Y. Zhang, G.H. Wang, J. Bao, W.W. Li, W. Zhang, L.L. Xu, D.F. Cai, PI3K/Akt pathway contributes to neurovascular unit protection of Xiao-Xu-Ming decoction against focal cerebral ischemia and reperfusion injury in rats, Evid. Based Complement. Alternat. Med. 2013 (2013) 459467–459482.
[17] R.A.B. Drury, E.A. Wallington, Preparation and fixation of tissues, in: R.A.B. Drury, E.A. Wallington (Eds.), Carleton's Histological Technique, fifth edition, University Press, Oxford, 1980, pp. 41–54.
[18] J.M. Elias, M. Margiotta, D. Gaborc, Senstivity and detection efficacy of the peroxidase antiperoxidase (PAP), avidin-biotin peroxidase complex (ABC), and peroxidase-labeled avidin-biotin (LAB) methods, Am. J. Clin. Pathol. 92 (1) (1989) 62–67.
[19] J.W. Tuckey, Comparing individual means in the analysis of variances, Biometrics 5 (1949) 99–114.
[20] T. Wang, S. Duan, H. Wang, S. Sun, B. Han, F. Fu, Neurological function following cerebral ischemia/reperfusion is improved by the Ruyi Zhenbao pill in a rats, Biomed. Rep. 4 (2016) 161–166.
[21] Z.S. Vexler, M. Yenari, Does inflammation after stroke affect the developing brain differently than adult brain? Dev. Neurosci. 31 (2009) 378–393.
[22] M. Godínez-Rubí, A.E. Rojas-Mayorquín, D. Ortuño-Sahagún, Nitric oxide donors as neuroprotective agents after an ischemic stroke-related inflammatory reaction, Oxidative Med. Cell. Longev. 5 (2013) 297357.
[23] M. Lin, W. Sun, W. Gong, Z. Zhou, Y. Ding, Q. Hou, Methylophiopogonanone A protects against cerebral ischemia/reperfusion injury and attenuates blood-brain barrier disruption in vitro, PLoS ONE 10 (4) (2015) e0124558.
[24] R.Z. Gomes, G.M.M. Romanek, M.P.D.C. Amaral, H.G.K. Akahane, Evaluation of the effect of allopurinol as a protective factor in post ischemia and reperfusion inflammation in Wistar rats, Acta Cir. Bras. 31 (2) (2016) 126–132.
[25] Y. Dan-juan, G. Hui-yang, Effect of propofol on mitochondrial ATP content and ATPase activity in hippocampus of rats with cerebral ischemia-reperfusion injury, Saudi Journal of Biological Sciences 24 (2017) 246–250.
[26] V.D. Ramirez, J. Zheng, K.M. Siddique, Membrane receptors for estrogen, progesterone, and testosterone in the rat brain: fantasy or reality, Cell. Mol. Neurobiol. 16 (1996) 175–198.
[27] J. Zheng, V.D. Ramirez, Rapid inhibition of rat brain mitochondrial proton F0F1-ATPase activity by estrogens: comparison with Na1, K1-ATPase of porcine cortex, Eur. J. Pharmacol. 368 (1999) 95–102.
[28] J. Zheng, V.D. Ramirez, Purification and identification of an estrogen binding protein from rat brain: oligomycin-sensitivity conferring protein (OSCP), a subunit of mitochondrial F0F1-ATP synthase/ATPase, J. Steroid Biochem. Mol. Biol. 68 (1999) 65–75.
[29] M. Diaz-Flores, L.A. Baiza-Gutman, N.N. Pedron, J.J. Hicks, Uterine glutathione

reductase activity: modulation by estrogens and progesterone, Life Sci. 65 (1999) 2481–2488.
[30] J. Wang, P.S. Green, J.W. Simpkins, Estradiol protects against ATP depletion, mitochondrial membrane potential decline and the generation of reactive oxygen species induced by 3-nitroproprionic acid in SK-N-SH human neuroblastoma cells, J. Neurochem. 77 (2001) 804–811.
[31] S. Duan, T. Wang, J. Zhang, M. Li, C. Lu, L. Wang, Y. Zou, F. Fu, Huatuo Zaizao pill promotes functional recovery and neurogenesis after cerebral ischemia-reperfusion in rats, BMC Complement. Altern. Med. 17 (2017) 19.
[32] Y. Zhao, P. Wang, S. Chen, C. Han, Q. Yan, L. Zheng, J. Jia, Z. Ren, X. Zhen, Dihydromyricetin protects against cerebral ischemia/reperfusion injury via suppressing microglia-mediated neuroinflammation and activation of ERK1/2-CREB signaling pathway, J. Funct. Foods 33 (2017) 76–84.
[33] T. Jover-Mengual, S.R. Zukin, A.M. Etgen, MAPK Signaling is critical to estradiol protection of CA1 neurons in global ischemia, Endocrinology 148 (3) (2007) 1131–1143.
[34] J. Fu, R. Xue, J. Gu, Y. Xiao, H. Zhong, X. Pan, R. Ran, Neuroprotective effect of calcitriol on ischemic/reperfusion injury through the NR3A/CREB pathways in the rat hippocampus, Mol. Med. Rep. 8 (2013) 1708–1714.
[35] Y. Dong, A. Jiang, H. Yang, H. Chen, Y. Wang, Phytoestrogen α-zearalanol improves memory impairment and hippocampal neurogenesis in ovariectomized mice, Sci. World J. 2014 (2014) 862019.
[36] S. Duan, T. Wang, J. Zhang, M. Li, C. Lu, L. Wang, Y. Zou, F. Fu, Huatuo Zaizao pill promotes functional recovery and neurogenesis after cerebral ischemia-reperfusion in rats, BMC Complement. Altern. Med. 17 (2017) 19.
[37] S. Yu, Q. Cheng, L. Li, M. Liu, Y. Yang, Y. Ding, 2-(4-Methoxyphenyl) ethyl-2-acetamido-2-deoxy-β-D-pyranoside confers neuroprotection in cell and animal models of ischemic stroke through calpain1/PKA/CREB-mediated induction of neuronal glucose transporter 3, Toxicol. Appl. Pharmacol. 277 (2014) 259–269.
[38] R. López-Grueso, J. Gambini, K.M. Abdelaziz, D. Monleón, A. Díaz, M. El Alami, V. Bonet-Costa, C. Borrás, J. Viña, Early, but not late onset estrogen replacement therapy prevents oxidative stress and metabolic alterations caused by ovariectomy, Antioxid. Redox Signal. 20 (2) (2014) 236–246.