Bull. Egypt. Soc. Physiol. Sci. *39(1)*, 52-62

Bull. of Egyp. Soc. Physiol. Sci.

(Official Journal of Egyptian Society for Physiological Sciences)

(pISSN: 1110-0842; eISSN: 2356-9514)

Does Oxytocin Have A Neuro-protective Impact in Rats' Stroke Model ?

Hayam G Sayyed[1], Eman S.H. Abd Allah[1], Mahmoud F. Sherif [2]

[1]Medical Physiology Department, [2] Pathology Department, Faculty of Medicine, Assiut University, Assiut, Egypt

Received: 16 Oct. 2018
Accepted: 26 Oct 2018
Available online: 1 Mar 2019

Keywords

- Ischemia/reperfusion
- Na^+/K^+–ATPase
- TNF-α
- Oxytocin
- Oxidative stress index

Abstract

Background: Stroke is a causative factor of disabilities and death. Various mechanisms involved in the cerebral ischemia-reperfusion pathophysiology, including oxidative stress along with inflammation. **Aim:** This research assessed the impact of oxytocin in lessening the detrimental effects of reperfusion in the cerebral ischemia/reperfusion (I/R) injury with the causal mechanisms. **Materials:** The cerebral ischemia-reperfusion injury was elicited by bilateral common carotid artery obstruction for 30 min followed by reperfusion for 24 h in rats. Forty eight rats were divided into: sham-operated group, oxytocin control group (underwent sham operation and given intraperitoneal oxytocin at a dose 750 µg/kg body weight), ischemia and reperfusion group and oxytocin-treated-ischemia and reperfusion group underwent I/R injury and given oxytocin 15 min before perfusion. Total antioxidant capacity, total peroxide, oxidative stress index, tumor necrosis factor-alpha and sodium/potassium-ATPase (Na^+/K^+-ATPase) level were measured in the cerebral homogenate. Histopathological analyses using H&E stain were carried out. **Results:** Administration of oxytocin lowered the ischemia-reperfusion-induced elevations in the cerebral total peroxide, oxidative stress index and tumor necrosis factor-alpha concentrations and increased total antioxidant capacity concentration and Na^+/K^+-ATPase level. Together, these changes were associated with alleviated histopathological alteration-induced by ischemia-reperfusion injury. **Conclusion:** Oxytocin has a neuro-protective impact against the deleterious effects of reperfusion via amelioration of oxidative stress, and inflammation and restoration of the declining level of the Na^+/K^+-ATPase. Thus, OT probably has a therapeutic impact on ischemic stroke.

Corresponding author: Dr. Hayam G Sayyed, **Mailing address:** Dr. Hayam G Sayyed, Medical Physiology Department, Faculty of Medicine, Assiut University, Assiut, Egypt, **Tel:** 01011826773, **Fax:** 002 088 2332278, **E-mail:** hayam_said3@aun.edu.eg

INTRODUCTION

Ischemic stroke is a major contributory cause to disabilities and death (1) that results from transient or permanent large cerebral arteries occlusion (2). At present, the main effectual treatment is immediate reperfusion which may aggravate the brain injury through a sequence of pathophysiologic mechanisms such as augmented excitatory neurotransmitters liberation, enhanced free radicals production, apoptosis and inflammation, although the precise mechanism is not entirely identified (3, 4).

Growing evidence reveals that inflammation has an essential role (2). Inflammation subsequent to stroke is initiated by quick production of numerous inflammatory cytokines including tumor necrosis factor-α (TNF-α) which is believed to has a pivotal role in the pathophysiology of ischemia/reperfusion-provoked brain damage (3).

Na^+/K^+–ATPase is an integral membrane protein that widely expressed in nervous system (5), that is abundant in synaptic membranes where it has a vital role in neurotransmission (4). Na^+/K^+–ATPase dysfunction has been implibral ischemia (5).

Oxytocin (OT), a neuropeptide synthesized mostly in the paraventricular and supraoptic nuclei (6), functions as a neuromediator, neurotransmitter or hormone, that controls various physiological processes as gestation and labor initiation, lactation, mother-baby synchrony, orgasm and copulation, stress suppression, temperature regulation, olfactory processing, visual contact and individual's recognition (7). OT can exert an anti-inflammatory effect, accelerate wound repair and repress stress-related immune diseases (8). Moreover, the protective contribution of OT aganist ischemia/reperfusion (I/R) injury has been demonstrated in the heart (11, 12), ovary and uterus (9) and kidney and liver (10).

This study proposed to investigate the neuro-protective effect of oxytocin administration in alleviating the potentially deleterious effects of reperfusion in rats through estimating the anti-oxidant, anti-inflammatory and Na^+/K^+-ATPase level in ischemic/reperfused rat brain.

MATERIALS AND METHODS

Chemicals

Oxytocin (Cat N: 04375, Sigma-Aldrich, St. Louis, MI, USA) was freshly prepared in saline 0.9% and given intraperitoneally (i.p.) at a dose of 750 µg/kg body weight (this is the best dose after our preliminary study), 15 min before reperfusion. Intraperitoneal administration of oxytocin was according to Padurariu et al. (11) who demonstrated an antioxidant effect of intraperioteal administration of oxytocin in rat cerebral cortex.

Experimental Animals

Forty-eight healthy male Wistar Albino rats weighing from 250 to 300 g were purchased from the Faculty of Medicine Animal house, Assiut University. Rats were kept in sanitary stainless steel cages (20x32x20 cm for every 4 rats). Rats were kept on conventional light/dark cycle, within a ventilated room with supplied diet and water. Rats left for a week to acclimatize before starting of the experimental procedures. The research protocol complied with the "Guidelines of Experiments on Animals" and was approved by the Ethics Committee of our institution (approval No: 17300241). Rats were cared according to the *Guide for the Care and Use of Laboratory Animals* (12).

Experimental Design

Rats were segregated into four groups (12 rats each) as follows:

1. Sham-operated group (SO): rats underwent the surgical procedure, but without bilateral common carotid artery occlusion (CCAO) and received saline i.p.
2. Oxytocin-control group (OT): rats underwent a sham operation and injected with oxytocin (750 μg/kg body weight; i.p).
3. Ischemia and reperfusion group (I/R): rats subjected to bilateral CCAO for 30 min followed by reperfusion for 24 h.
4. Oxytocin-treated-ischemia and reperfusion group (I/R-OT): rats underwent I/R injury and received oxytocin (750 μg/kg body weight; i.p.) 15 min before reperfusion.

Cerebral ischemia was provoked by bilateral CCAO as previously portrayed by Spray et al. (16). Briefly, rats were anesthetized by 1% sodium pentobarbital (50 mg/kg, i.p.) (17). The neck was washed utilizing ethanol 70%. A midline neck incision was done; then, the CCAs were carefully isolated to protect the vagi. The carotid arteries were clamped by the bulldog for 30 min. Afterward, reperfusion started with the bulldog removal from the arteries; reflow of blood was confirmed by visual inspection of the arteries. Closure of surgical incisions was done with 6-0 sutures. Sham-operated rats underwent through a similar procedure without CCAO. During operation, a heating lamp was used to keep the rat warm. Aseptic procedures were maintained throughout the operation. Following surgery, rats have resided individually with a free supply of water and food.

At 24 h after CCAO or sham surgery, rats were euthanized with a lethal dose of pentobarbital (100 mg/kg body weight) (18). Then, brains were removed rapidly, weighed, frozen using liquid nitrogen, afterward stored at –80 °C until the biochemical determinations.

Biochemical Analyses

Six brains from each group were used for histopathology and the other six were rinsed with cold saline, blotted with filter paper, weighed and the cerebral cortex was homogenized in ice-cold phosphate-buffered saline (PBS; pH 7.4) by Glas-Col Homogenizer AQ5 and centrifuged at 10000 rpm for 10 min by ultra-centrifuge (Hettich EBA 12) at about 4°C and stored till used.

Cerebral T-AOC and TP Estimation

Total antioxidant capacity (T-AOC) was measured colorimetrically using the commercially available kit (Bio-Diagnostics, Giza, Egypt) according to the method of Koracevic et al. (13). The cerebral cortex supernatant antioxidants eliminate a definite amount of hydrogen peroxide (H_2O_2). The residual H_2O_2 is determined colorimetrically by an enzymatic reaction which involves the conversion of 3,5-dichloro-2-hydroxy benzene sulphonate to a colored product. Total peroxide (TP) was determined as portrayed by Harma et al. (14). Briefly, it is determined colorimetrically by an enzymatic reaction which involved the oxidation of xylenol orange into a colored product. Oxidative stress index (OSI), an indicator for oxidative stress was calculated as the percentage ratio of TP to TAC in mM/L (14).

Cerebral TNF-α Estimation

Enzyme-linked immunosorbent assays kit was used to measure tumor necrosis factor-alpha (TNF-

α) concentration (Cat. No: K0331196, Koma Biotech), according to the instructions supplied with the kit.

Cerebral Na^+/K^+–ATPase

Sodium/potassium adenosine triphosphatase (Na^+/K^+–ATPase) level was estimated using spectrophotometry as previously reported by Sehirli et al. (15).

Total Protein Evaluation

Protein concentrations were measured using the Lowry et al. (16) method with the bovine serum albumin as a standard.

Histopathology

The cerebral cortex was removed, fixed in 10% formalin, embedded in paraffin, cut into sections (4 μm in thickness), deparaffinized, and stained with hematoxylin and eosin (H&E). The histological examination was performed under a light microscope.

Statistics

Results are expressed as the mean ± standard deviation (SD). Differences in the means of variables were analyzed by non-parametric Kruskal-Wallis H test, and then multiple comparisons were done using the Mann–Whitney U test after normality was checked by Shapiro-Wilk test. A value of $P \leq 0.05$ was considered statistically significant. Spearman's correlations between Na^+/K^+-ATPase level (dependent variable) and OSI and TNF-α (independent variables). All analyses were carried out with SPSS version 21 (Chicago, Illinois, USA).

RESULTS

Effect of oxytocin on the cerebral T-AOC, TP, and OSI ratio

Figure1 shows the cerebral concentrations of T-AOC and TP and OSI ratio. The I/R group reveals a significant decline in the T-AOC concentration compared with the sham-operated and OT groups ($P<0.05$ for each). Interestingly, OT administration in the I/R-OT group elevates significantly the T-AOC ($P<0.05$ versus I/R group). In contrast, the I/R group reveals a significant increase in the TP concentration compared with the sham-operated and OT groups ($P<0.05$ and $P<0.01$; respectively). Compared with the I/R group, OT administration reduces significantly the TP concentration ($P<0.05$). Similarly, the I/R group shows a significant increase in OSI ratio compared to OT group ($P<0.01$). Meanwhile, OT administration decreases OSI ratio ($P<0.05$) compared to the I/R group. This indicates that OT attenuates the oxidative stress in the cerebral I/R.

Effect of oxytocin on the cerebral TNF-α

Figure 2. displays the cerebral TNF-α concentration. TNF-α concentration increased significantly in the I/R group compared with the sham-operated and OT groups ($P<0.05$ and $P<0.01$; respectively). Fascinatingly, compared with the I/R group, OT administration reduces significantly the TNF-α concentration ($P<0.05$). This points to the impact of OT in suppressing the neuro-inflammatory reaction in the cerebral I/R.

Effect of oxytocin on the cerebral Na^+/K^+-ATPase

Figure 3 shows the cerebral Na^+/K^+–ATPase level. The I/R group exhibits a significant decline in the Na^+/K^+–ATPase level compared with the sham-operated and OT groups ($P<0.05$ for each).

Compared with the I/R group, OT administration in the I/R-OT group restores the declining level of the Na^+/K^+–ATPase (P<0.05).

Correlation analyses

Figures 4 and 5 show significant negative correlations between the Na^+/K^+-ATPase and the oxidative stress index and TNF-α; respectively.

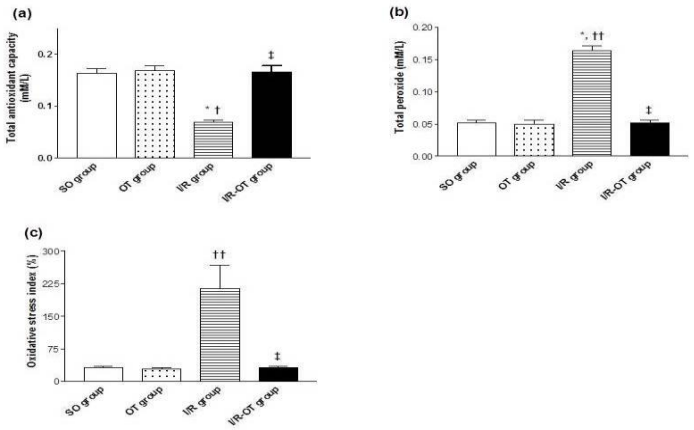

Figure 1. Effect of OT administration on the cerebral concentrations of T-AOC (a) and TP (b) and OSI ratio (c). Values are represented as the mean ± SD. *: P<0.05 significance difference from the sham-operated group, †: P<0.05 and ††: P<0.01 significance difference from the OT group and ‡: P<0.05 significance difference from I/R group. Abbreviations: I/R, Ischemia and reperfusion; I/R-OT, Oxytocin-treated-ischemia and reperfusion; OSI, oxidative stress index; OT, Oxytocin-control; SO, sham-operated; T-AOC, total anti-oxidant capacity; TP, total peroxide

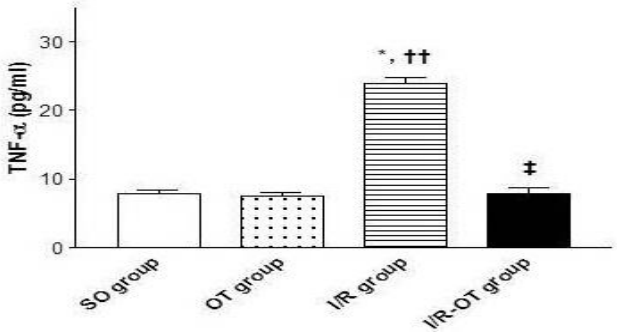

Figure 2. Effect of OT administration on the cerebral concentration of TNF-α. Values are presented as the mean ± SD. *: P<0.05 significance difference from the sham-operated group, ††: P<0.01 significance difference from the OT group and ‡: P<0.05 significance difference from I/R group. Abbreviations: I/R, Ischemia and reperfusion; I/R-OT, Oxytocin-treated-ischemia and reperfusion; OT, Oxytocin-control; SO, sham-operated; TNF-α, tumor necrosis factor alpha

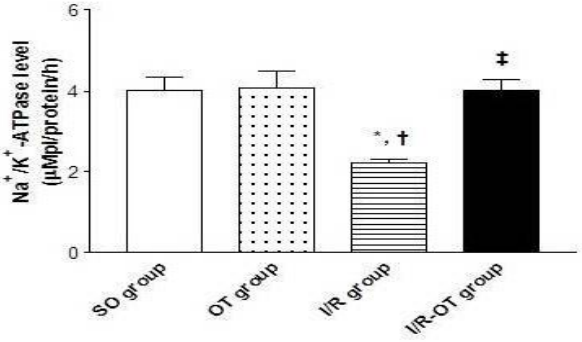

Figure 3. Effect of OT administration on the cerebral Na^+/K^+-ATPase level. Values are presented as the mean ± SD. *: P<0.05 significance difference from the sham-operated group, †: P<0.05 significance difference from the OT group and ‡: P<0.05 significance difference from I/R group. Abbreviations: I/R, Ischemia and reperfusion; I/R-OT, Oxytocin-treated-ischemia and reperfusion; OT, Oxytocin-control; SO, sham-operated

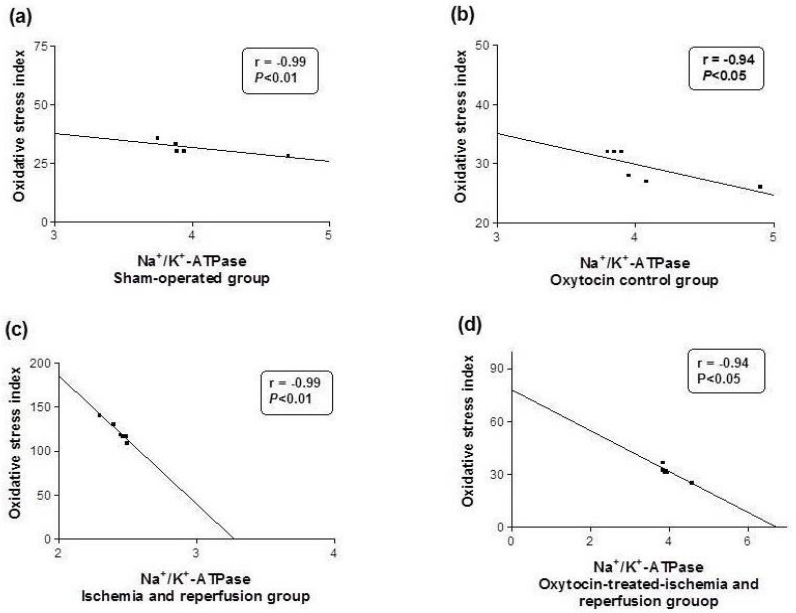

Figure 4. Correlation between the Na^+/K^+-ATPase level (dependent variable) and the oxidative stress index (independent variable). (a) sham-operated group, (b) oxytocin-control group, (c) ischemia and reperfusion group and (d) oxytocin-treated-ischemia and reperfusion group.

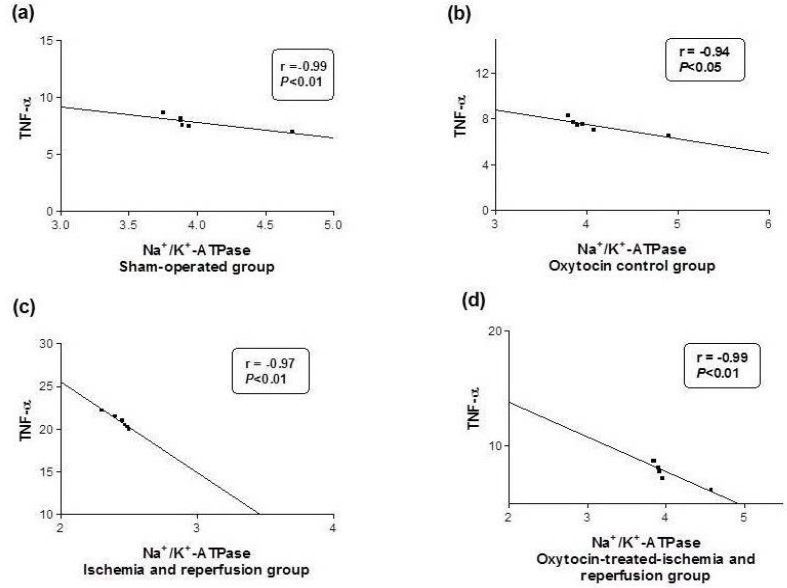

Figure 5. Correlation between the Na^+/K^+-ATPase level (dependent variable) and the tumor necrosis factor-alpha (TNF-α) concentration (independent variable). (a) sham-operated group, (b) oxytocin-control group, (c) ischemia and reperfusion group and (d) oxytocin-treated-ischemia and reperfusion group.

Histopathology

Cortical sections of the sham-operated (Figure 6A) and oxytocin control (Figure 6B) groups, stained with H &E, reveal that the structure of the neurons is preserved with average-sized round nuclei plus amphophilic cytoplasm. I/R-injury in ischemia/reperfusion group results in ischemic changes in the form of shrunken cell size, nuclei pyknosis and enhances cytoplasmic eosinophilia (Figure 6C). Figure 6D shows that oxytocin mitigates the ischemic changes-induced by I/R-injury with averagely sized neurons in oxytocin-treated-ischemia/reperfusion group.

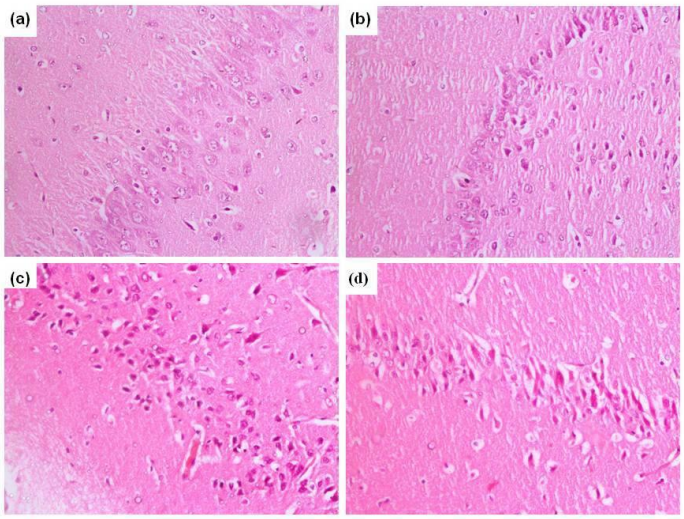

Figure 6. Hematoxylin and Eosin staining of the brain sections. (a): sham-operated group shows preservation of neurons abundant amphophilic cytoplasm, round nuclei and prominent nucleoli (H&E, 400x). (b): oxytocin-control group shows unaffected neurons shows preservation of normal-looking neurons (H&E, 400x). (c): ischemia and reperfusion group shows ischemic changes in the form of shrunken cellular size, nuclei pyknosis and increases cytoplasmic eosinophilia (H&E, 400x). (d): oxytocin-treated-ischemia and reperfusion group shows mild cellular shrinkage and nuclear change (H&E, 400x).

DISCUSSION

Ischemia results in a widespread disturbance of the cerebral biochemical system that is amplified by reperfusion (23). Our research revealed that OT administration to ischemia/reperfusion injury exerted a neuro-protective effect via suppressing oxidative stress, and attenuating inflammatory response; these findings are supported by histopathology. This suggested its possible importance as a veritable therapeutic attribution in the cerebral ischemia.

Oxidative stress incriminated as one of the early and the vital damaging consequence of blood flow restoration to the ischemic areas (24, 25). Oxidative stress is marked by free radical overproduction, antioxidant reduction with lipid peroxidation (17). Owing to low antioxidants, high peroxidizable lipids, elevated oxygen expenditure and elevated iron concentration which operate as pro-oxidants in pathological states, the brain is very vulnerable to damage via reactive oxygen species (ROS) (24). ROS are extremely unsteady and interact with cell biomolecules as lipid, protein, and DNA resulting in impairment of membrane integrity, enzymatic action and genomic steadiness (26, 27). Our results of elevation of the TP concentration and OSI ratio along with reduction of the T-AOC concentration in I/R rats are consistent with the finding of Al Dera (18) and Jia et al. (19). Furthermore, our research revealed that OT treatment suppressed oxidative stress via restoring the declined the T-AOC concentration, attenuating the increased TP concentration and reducing the OSI ratio. Similarly, Karelina et al. (17) demonstrated that OT reduced rat neuronal damage by enhancing antioxidant activity and diminishing oxidative stress. Furthermore, Erbas et al. (29) demonstrated that OT had an impact on diabetic neuropathy by repressing oxidative stress. Additionally, the OT anti-oxidant capacity has been reported in the renal (20) and the hepatic (21) I/R models.

The antioxidant effect of OT-mediated neuroprotection claimed to free radical scavenging, lipid peroxidation reduction and

NADPH-dependent superoxide activity attenuation (17).

Subsequent cerebral I/R, release of the pro-inflammatory cytokines from the activated endothelial cells, recruited leukocytes and occupant cells in the brain, comprising microglia and neurons (32) results in disruption of the blood–brain barrier integrity and fluid accumulation leading to cell death (1). A number of studies reported that inflammation is essential for the ischemic progression (33, 34). In the agreement, this study demonstrated elevation of the TNF-α concentration in the I/R rats. Additionally, we revealed that OT lowered the I/R-induced elevation in the TNF-α concentration. Similarly, Ragy and Aziz (10) revealed that OT ameliorated inflammatory reaction in the renal I/R. Yuan et al. (35) demonstrated that OT had anti-inflammatory effect against lipopolysaccharide-induced neuro-inflammation in mice. Thus, OT via the mitigation of inflammatory reaction probably has a valuable impact in the improvement of stroke.

Notably, the attenuation of oxidative stress came with the reduction of pro-inflammatory cytokines. Thus, it is probable that the OT-mediated neuroprotection is partly arbitrated to its anti-inflammatory effect and antioxidant properties.

Na^+/K^+–ATPase activates the counter-transport of Na^+ and K^+ ions (22) and regulates the activity of gamma aminobutyric acid (4) and glutamate (23) transporters. Na^+/K^+–ATPase dysfunction resulted in the influx of Na^+, Ca^{+2} and Cl^- ions and efflux of K^+ ions. These lead to increased water uptake from the extracellular fluid and cytotoxic cerebral edema (5). We concurred with the results of Ilesanmi et al. (23) who found reduced Na^+/K^+–ATPase level following cerebral I/R. We extended the finding to the restoration of the Na^+/K^+–ATPase level following OT administration. Girardet et al. (24) reported that OT exposure increased synthesis of alpha and beta subunits of Na^+/K^+–ATPase.

Furthermore, we revealed an inverse association of the Na^+/K^+–ATPase level with OSI ratio. Thus, the decline of Na^+/K^+–ATPase level could claim to oxidative stress. This result is concurred with Yan et al. (25) finding who demonstrated that increasing Na^+/K^+–ATPase activity attenuated oxidative stress-provoked myocardial damage. It was revealed that ROS motivated Na^+/K^+–ATPase endocytosis (26), carbonylated the α1 subunit of Na^+/K^+–ATPase (27). Recently, it was found that the Na^+/K^+–ATPase, and ROS form a feed-forward cycler as increasing ROS, in feedback, stimulated the Na^+/K^+–ATPase endocytosis (25).

Also, we demonstrated an inverse relation between the Na^+/K^+–ATPase level and TNF-α concentration. This finding has coincided with the result of Kobayashi et al. (42) who reported that reduction of the Na^+/K^+–ATPase enhances the cardiac inflammation. Furthermore, Zhang et al. (28) found that embarrassment of the Na^+/K^+–ATPase enhanced the TNF-α expression and cardiac dysfunction.

In conclusion, this study revealed a neuro-protective impact of OT in alleviating the potentially deleterious effects of reperfusion in rats through amelioration of oxidative stress and inflammation, and restoration of the declining level of the Na+/K+–ATPase.

Limitations and future work

Small number of rats was used in each group. Moreover, excitotoxicity was not measured. Therefore, our future work will include larger number of rats and the impact of oxytocin on excitotoxicity will be measured. Also, we will estimate the impact of different doses of oxytocin on different reperfusion times.

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