CHAPTER 7

IN-VIVO EVALUATION OF NEUROPROTECTIVE POTENTIAL OF PROLACTIN

Chapter Highlights

- ***Prolactin is a neuropeptide known for its pleiotropic actions in brain***
- ***Though prolactin has shown neuroprotection against glutamate excitotoxicity in-vitro, its in-vivo effects are yet to be studied***
- ***The chapter discusses effect of prolactin on physiological and biochemical parameters of ischemic mice***

ABSTRACT

Lack of available therapeutics for cerebral ischemia necessitates a dire need for designing novel strategies for combating ischemic pathophysiological cascade. In such scenarios, neuroprotective strategies have emerged as a possible successful approach to ameliorate cerebral ischemic conditions. The neuropeptide prolactin is a pleiotropic hormone which affects various physiological conditions, specifically the ones concerning the central nervous system. Previous scientific studies suggest that prolactin can combat neurotoxicity, neuronal stress and provide neuroprotection to hippocampal neurons *in-vitro*. The present study explores the ability of this neuropeptide in conferring *in-vivo* neuroprotection in global cerebral ischemia model of rat. Also, the study tries to optimize the dose of prolactin which will be effective for conferring neuroprotection in cerebral ischemic condition. The results revealed that prolactin significantly reduces cerebral infarct, brain water content and restores the physiological conditions like blood pressure, heart rate and cerebral blood flow. Also, prolactin markedly reduces the increased levels of the neurotransmitters GABA and glutamate in

different brain parts of ischemic rats. Cerebral calcium and nitrate concentrations were also successfully restored by prolactin post-treatment. The ability of prolactin in effectively combating the overall pathogenesis occurring in brain due to ischemia-reperfusion injury establishes the molecule as a probable candidate for further therapeutic development against cerebral ischemia.

7.1. INTRODUCTION

Acute cerebrovascular disease generally occurs due to interruption in cerebral blood flow and can be either ischemic or hemorrhagic [1]. An obstruction in cerebral blood flow deprives the brain of oxygen, glucose and other nutrients necessary for normal brain functions [1,2] and results in triggering of an ischemic cascade which involves loss of ionic homeostasis and subsequent neurotransmitter release [2]. Cerebral ischemia happens to be a global burden causing death and permanent disability [3, 4] with lack of specific therapeutic treatment [1]. The only approved therapy for combating cerebral ischemia is recombinant tissue plasminogen activator (rtPA) [1, 2] which is associated with the risk of increasing brain hemorrhage [2]. In such scenarios, recent scientific research has focused on neuroprotective therapies for combating the ischemic pathophysiology by inhibiting the cellular pathways associated with the ischemic insult [5]. By inhibiting one or more intermediates of the ischemic pathway, neuroprotective agents are able to prevent or delay the neuronal injury. Prolactin (PRL) is a peptidic hormone which induces behavioral changes like maternal behavior and anxiety reduction [6, 7]. PRL also induces neurogenesis [8], activates glial cells [9], causes proliferation of hippocampal precursor cells [10] and also provide neuroprotection against glutamate and kainic acid excitotoxicity [11-13]. It has also been established that neuroprotection exhibited by PRL in hippocampus is mediated via its receptors [12]. A recent

study establishes neuroprotective action of PRL against glutamate excitotoxicity in primary culture of rat hippocampal cells is due to PRL's ability to maintain calcium ion homeostasis and up-regulation of Bcl-2 expression [14]. Though neuroprotective ability of PRL against glutamate excitotoxicity has been demonstrated *in-vitro*, no known study has yet reported PRL's therapeutic ability as a neuro-protectant against cerebral ischemia *in-vivo*.

The present study attempts to evaluate the ability of PRL in conferring neuroprotection in global cerebral ischemia in rat model. Global cerebral ischemia is induced by bilateral common carotid artery occlusion (BCCAO) which is a frequently used model for studying neuroprotection in rodents [15,16]. BCCAO model has also been widely used to study the pathophysiological changes occurring due to induced cerebral ischemia [15-17].

7.2. MATERIALS AND METHODS

7.2.1. Dose preparation of Prolactin and administration

PRL was purchased from Sigma-Aldrich (Cat. No.: L6520; Prolactin from sheep pituitary) and different concentrations were prepared by dissolving in normal saline solution to obtain final doses of 1mg/Kg, 0.5mg/Kg and 0.1mg/Kg, which for further references are referred as Treated-A, Treated-B and Treated-C respectively. The doses were administered intra-nasally and each nostril received 5 µL of the prolactin solution. The vehicle group received 5 µL of saline solution through each nostril.

7.2.2. Inducing Global Cerebral Ischemia by BCCAO

Male inbred albino rats of weight of 150±25 grams were randomly divided into five groups, viz., Sham, Vehicle, Treated-A, Treated-B and Treated-C (n=15 for each group). All the animals except for Sham group were subjected to BCCAO surgical procedures according to

the method reported by Wang *et al* [18] after being anesthetized with a combination of xylazine (10 mg/kg b.w.) and ketamine (50 mg/kg b.w.). Common carotid arteries or CCAs were identified after the neck skin was opened via a vertical midline-incision (~ 1.5 cm length) and both the left and right CCAs were ligated with a cotton suture (5-0) after careful dissociation of vagal nerves from respective CCAs. The incision was stitched and the animals were returned to their respective cages at room temperature (25±2°C). Reperfusion was performed by cutting off the ligature cautiously by a surgical blade after 1h of occlusion and was maintained for 4 hrs before euthanasia. Throughout the surgical procedure, the rectal temperature of the animals was maintained at a constant 37±0.5°C. The sham group of animals underwent similar surgical procedures except for the ligature of CCAs. The workflow is provided in Fig. 7.1.

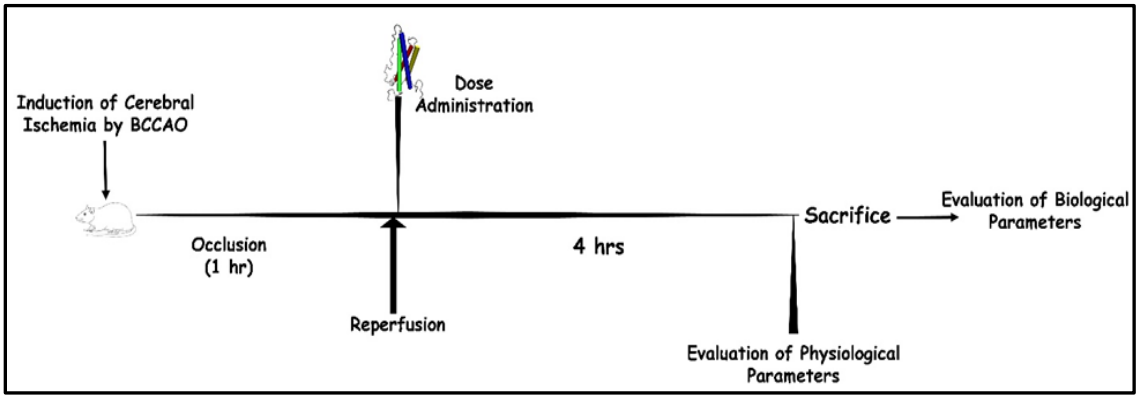

Fig. 7.1.: Experimental workflow for cerebral ischemia induction and PRL administration.

7.2.3. Evaluation of physiological parameters

Disruption of regional cerebral blood flow (rCBF) remains major reason behind cerebral ischemia [19, 20]. Also, transient elevation in mean arterial pressure and heart rate are marked characteristics of cerebral ischemia [21,22]. For the present study, systolic blood pressure (SBP) and heart rate of the animals were measured using tail cuffs supported with piezoelectric

sensors along with NIBP controller (AD Instruments) and rCBF was measured at co-ordinates by 6 mm lateral and 1 mm posterior to bregma on both the hemispheres Blood Flow Meter (AD Instruments) using the principles of Laser Doppler Flow. Animals with CBF reduced to 80% were only included in the study. Also, CBF was also monitored throughout the study till the end of 4 hrs reperfusion.

7.2.4. Biochemical parameter Estimation

To understand the effect of prolactin in combating cerebral ischemic conditions, concentration of biochemical markers in different brain regions, i.e., cortex, cerebellum and hippocampus were estimated. For this study, 25 male rats were divided into five groups (n=5) namely, Sham, Vehicle, Treated-A, Treated-B and Treated-C.

7.2.4.1 Estimation of Glutamate and ɣ-Aminobutyric acid (GABA) concentrations

Increase in the concentrations of the major neurotransmitters Glutamate and GABA is a hallmark of cerebral ischemia [2, 23, 24]. Estimation of Glutamate and GABA concentration in different brain regions of each group was performed using HPLC-UV method as described in the section 4.2.9.

7.2.4.2. Determination of Calcium concentration

Calcium concentrations in different brain regions for animals in every group were measured using calcium detection kit (Abcam, CatNo.: ab102505) according to the supplied protocol. The experiment was performed as per the procedure described in section 4.2.10.

7.2.4.3. Nitrate concentration Estimation

Nitrate concentration in various brain region was evaluated as an indirect estimation of changes in nitric oxide (NO) concentration, since the latter is highly unstable [25, 26]. The nitrate concentration in cortex, cerebellum and hippocampus of animals for every group was carried out according to the protocol described in 4.2.11.

7.2.5. Analysis of Brain Parameters

7.2.5.1. Infarction volume Estimation

Cerebral infarction volume of all five groups (n=5) were measured using tri-phenyl-tetrazolium chloride (TTC) staining as per the procedure decribed in section 4.2.6.

7.2.5.2. Study of Brain Edema

Cerebral ischemia leads to accumulation of water in brain tissues, thereby causing brain swelling [27]. Cerebral edema of animals for all the groups (n=5 for each group) were measured by quantifying brain water percentage using the wet and dry-weight method as decribed in section 4.2.8.

7.2.6. Cell death analysis by Annexin-FITC/PI staining

Effect of PRL on ameliorating cell death was analyzed using Annexin V Kit: sc-4252 AK (Santa Cruz Biotechnology, USA). Brain cortical tissue for each group (n=4) was dissociated to single cell suspension (1×10^6 cells/mL) and stained according to the given protocol.

7.2.7. Statistical analysis

All the data sets were represented as Mean±S.D. and were statistically analyzed with one-way analysis of variance (ANOVA). $P<0.05$ were considered as statistically significant.

7.3. RESULTS

7.3.1. Higher doses of PRL restore physiological conditions

Physiological conditions were monitored throughout the experimental procedures and values recorded after 4 hrs of reperfusion following 1 hr occlusion were compared with the values observed in sham group after similar time duration (Fig.2 A-C). It was found that, blood pressure and heart rate of the animals of vehicle group significantly increased ($p < 0.001$) whereas there was a significant decrease ($p < 0.001$) in rCBF as compared to the sham group animals after 1 h occlusion and 4 h reperfusion (Fig.7.2. A-7.2.C). Interestingly, it was observed that animals of all three treated groups had their SBP restored ($p < 0.001$) (Fig. 7.2.A). Heart rate was restored in Treated-A and Treated-B group ($p < 0.001$ and $p < 0.01$ respectively) in comparison to vehicle group. After 4hr reperfusion following 1 hr occlusion, a significant restoration in CBF was also exhibited in Treated-A and Treated-B group animals ($p < 0.001$), but no significant difference in heart rate and rCBF was observed in animals of Treated-C group (7.2.B).

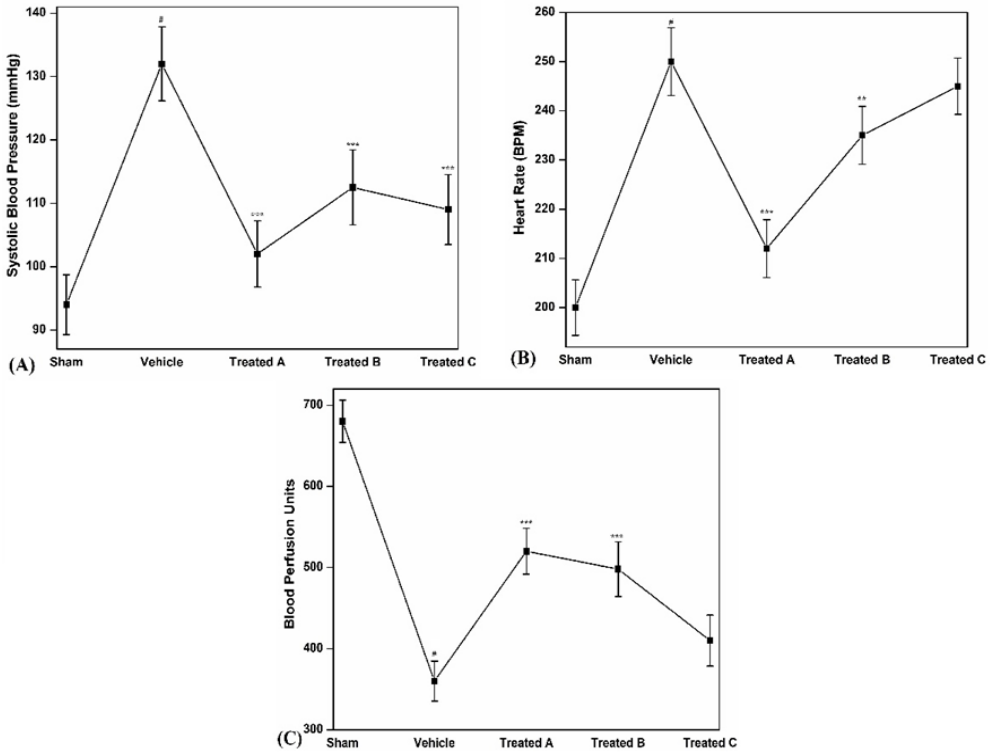

Fig. 7.2.: Changes in physiological parameters due to PRL post-treatment (after 1 hr occlusion followed by 4 hrs of reperfusion); (A) Systolic Blood Pressure, (B) Heart Rate and (C) Cerebral Blood Flow for different groups of animals. (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis; * signifies p < 0.001 and ** signifies p<0.01 compared to vehicle group; # signifies p < 0.001 of vehicle group as compared to sham).**

7.3.2. Restoration of neurotransmitter levels by PRL

Animals in Treated-A group showed significant restoration of brain glutamate in cortex and hippocampus in comparison to Vehicle group animals ($p < 0.001$) (Fig. 7.3.a). In cerebellum region of Treated-A group, glutamate percentage was also restored significantly ($p < 0.01$) (Fig. 7.3.a.). Treated-B group showed restoration of glutamate in cortex region ($p < 0.01$), but did not have any significant effect on cerebellum and hippocampus region. No significant difference in glutamate levels was observed in Treated-C group animals when compared to vehicle group. GABA level in cortex and hippocampus was significantly reduced in animals

of Treated-A group ($p < 0.001$) (Fig.7.3.b.). In cerebellum of Treated-A group animals also, a significant reduction of GABA level was revealed ($p<0.05$) (Fig. 7.3.b.). No significant differences in GABA levels were observed in any cerebral region of Treated-B and Treated-C groups in comparison to vehicle group animals.

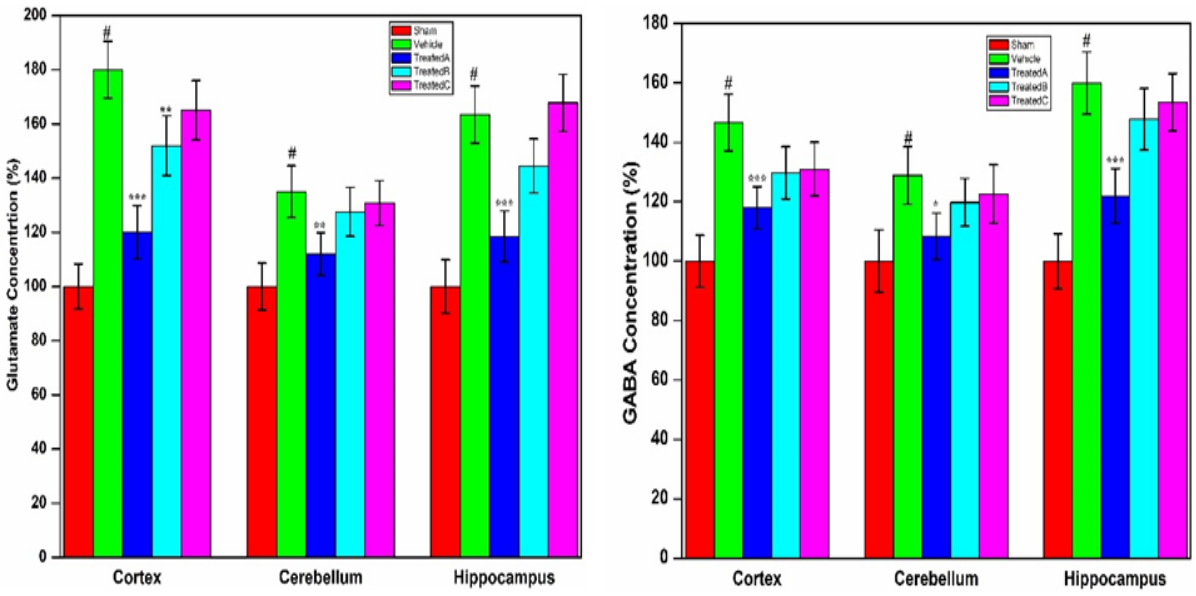

Fig. 7.3.: Effect of different doses of PRL on changes in neurotransmitter levels (a) Glutamate and (b) GABA in different cerebral regions. (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis; * signifies $p < 0.001$, ** signifies $p<0.01$ and * signifies $p < 0.05$ compared to vehicle group; # signifies $p < 0.001$ of vehicle group as compared to sham).**

7.3.3. PRL restores brain calcium concentration

Calcium estimation performed in three different cerebral regions revealed that prolactin administration has significant impact on the brain calcium level. In vehicle models, the calcium level in all three regions increased significantly as compared to sham group ($p < 0.001$) (Fig. 7.4.). The levels were considerably restored in all the Treated-A and Treated-B groups in all three cerebral regions ($p < 0.001$). Treated-C group also exhibited reduced calcium level in

hippocampus ($p < 0.001$) but did not show any significant difference in cortex and cerebellum regions.

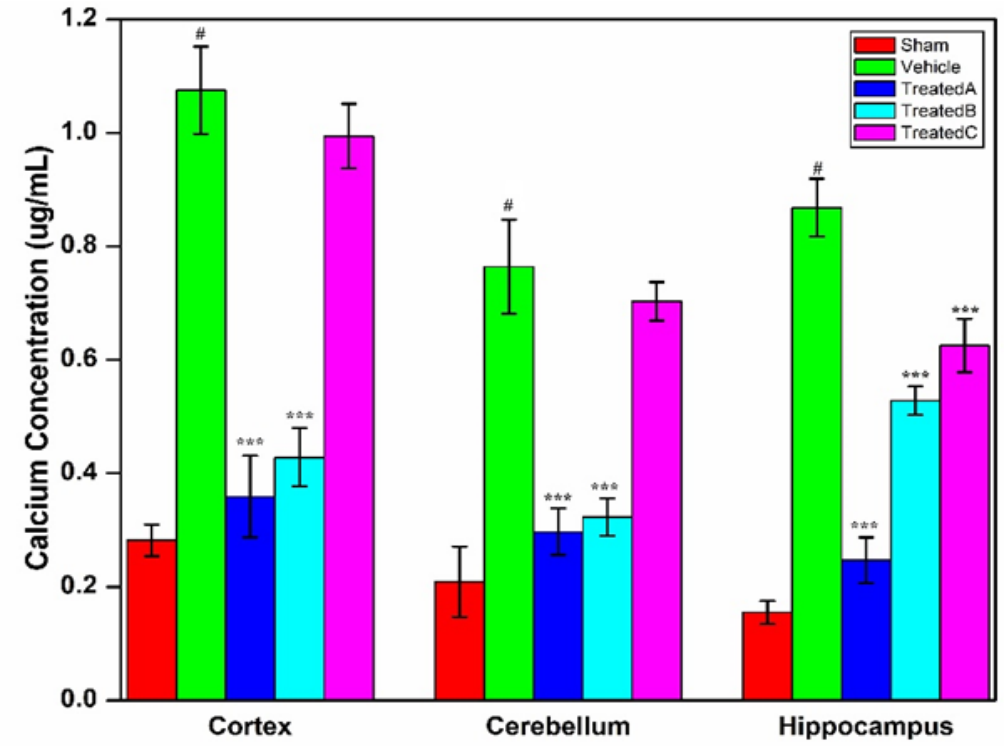

Fig. 7.4.: Restoration of calcium levels in different brain parts upon PRL treatment. (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis; * signifies $p < 0.001$; # signifies $p < 0.001$ of vehicle group as compared to sham).**

7.3.4. Cerebral nitrate levels were significantly reduced by PRL treatment

Highest dose of PRL (1mg/kg) effectively reduced ischemia induced elevated nitrate levels in all three different regions of rat brain (cortex: $p < 0.001$; cerebellum and hippocampus: $p < 0.01$) (Fig. 7.5.). Animals in Treated-B and Treated-C groups showed restored nitrate levels in cortex ($p < 0.01$) but no significant difference was observed in other brain regions (cortex and cerebellum) in Treated-C group.

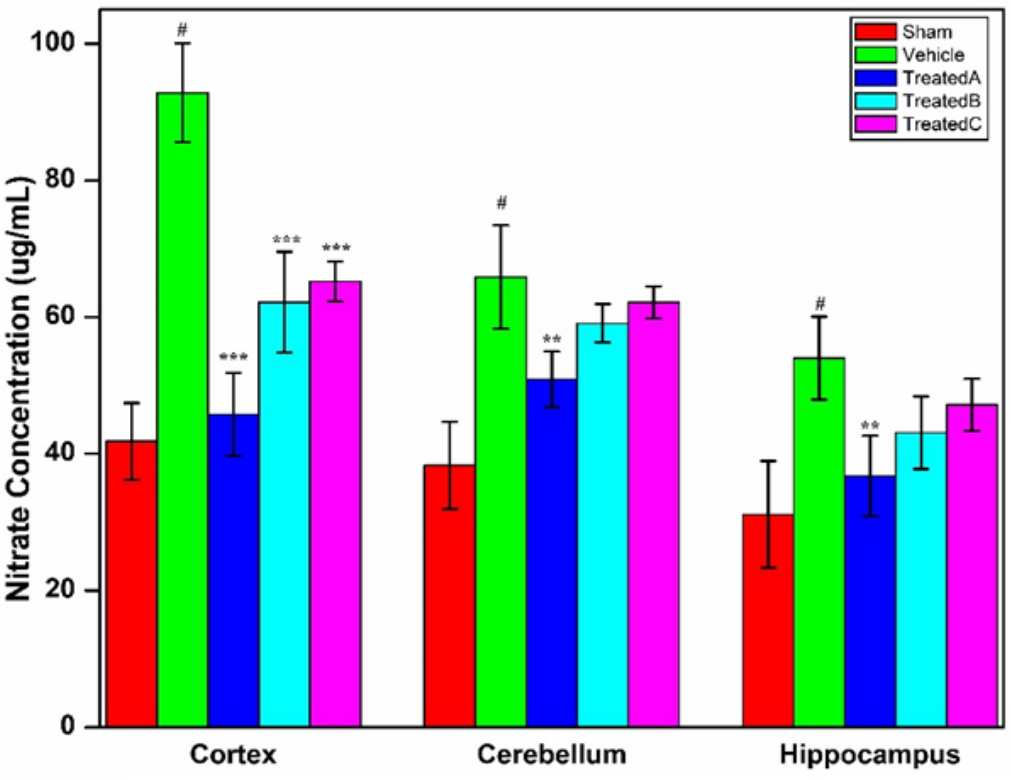

Fig. 7.5.: Effect of different doses of PRL on nitrate levels in various brain regions (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis; * signifies $p < 0.001$, ** signifies $p < 0.01$ compared to vehicle group; # signifies $p < 0.001$ of vehicle group as compared to sham).**

7.3.5. PRL Treatment decreases infarction volume

Cerebral infarction in rats of vehicle group was observed to be 15.5%, whereas that in Treated-A group animals was found to be 2.6%. There was a reduction of cerebral infarction by 83.2% in Treated-A group animals as compared to rats of vehicle group ($p < 0.001$). Infarction volume in animals of Treated-B and Treated-C was reduced by 61.8% and 59.1% in comparison to brain infarction of vehicle group animals ($p < 0.001$). There was no significant difference between infarction volumes of animals in Treated-B and Treated-C groups (Fig. 7.6.).

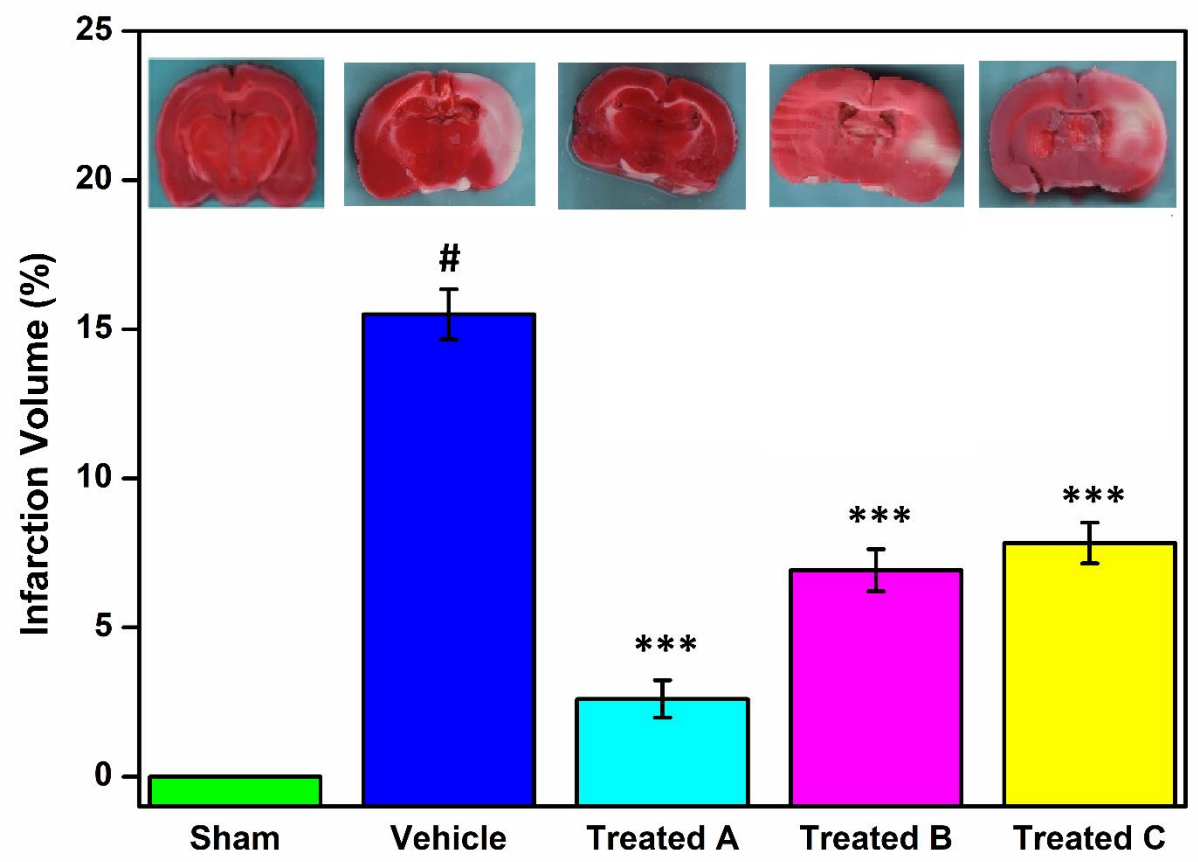

Fig. 7.6.: Dose dependent reduction of cerebral infarction as an effect of PRL treatment (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis; * signifies $p < 0.001$ compared to vehicle group; # signifies $p < 0.001$ of vehicle group as compared to sham).**

7.3.6. Cerebral edema was reduced due to PRL treatment

Increase in brain water content by 23.8% was detected in vehicle group as compared to animals in sham group which was subsequently decreased by 21.3% in Treated-A group in reference to vehicle group ($p < 0.001$) (Fig. 7.7). Cerebral edema was also reduced by 14.7% ($p < 0.001$) and 3.62% (no significant difference) in Treated-B and Treated-C group, respectively, as compared to vehicle group animals (Fig. 7.7.).

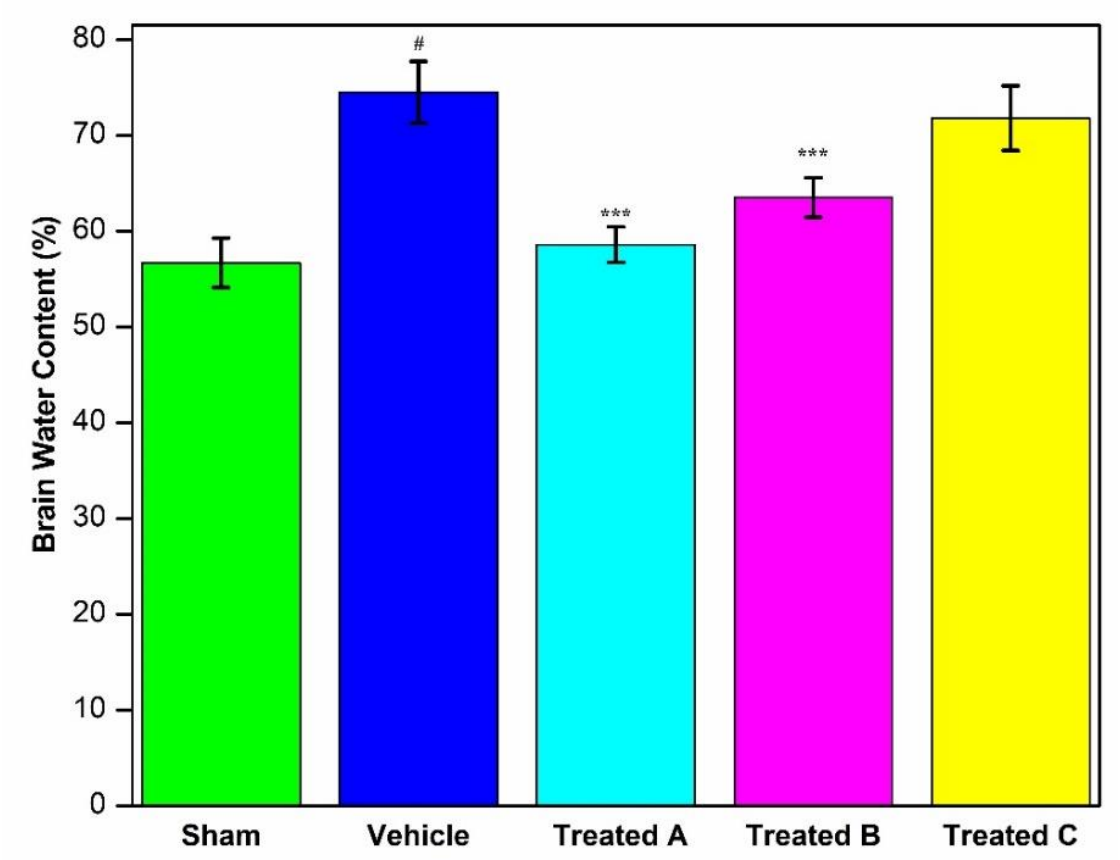

Fig. 7.7.: Reduction of cerebral edema with treatment of different doses of PRL (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis; * signifies $p < 0.001$ compared to vehicle group; # signifies $p < 0.001$ of vehicle group as compared to sham).**

7.3.7. PRL treatment ameliorates cell death in cortical region

An increased number of Annexin-FITC and PI positive cells were observed in vehicle group animals as compared to the sham group animals, confirming apoptosis and necrosis. All the three doses of PRL (0.1 mg/kg, 0.5 mg/kg and 1 mg/kg) exhibited marked reduction in number of apoptotic and necrotic cells. Among the three doses, lowest number of Annexin-FITC and PI positive cells were observed in Treated A (1 mg/kg) (Fig. 7.8.).

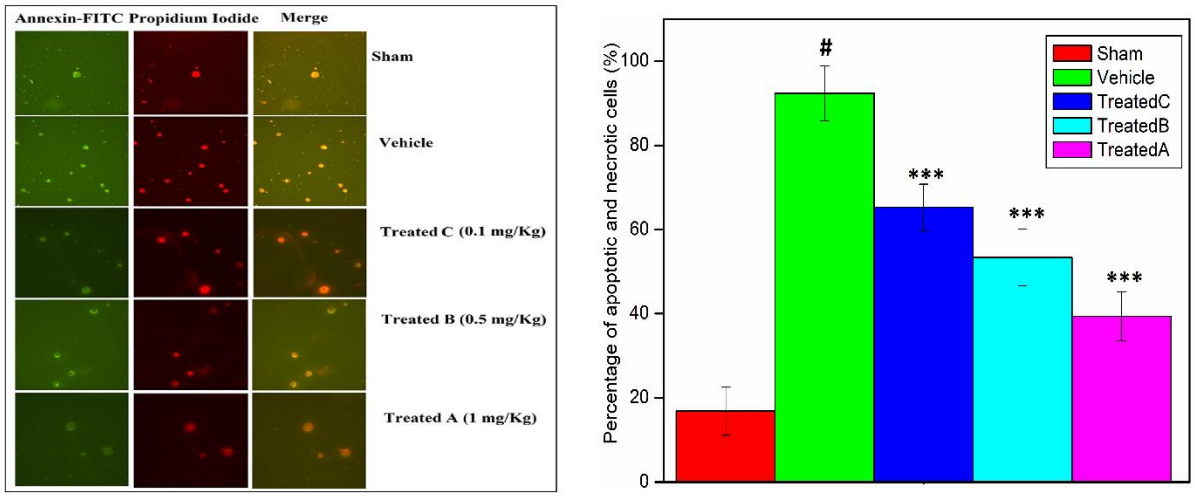

Fig. 7.8.: Effect of different doses of PRL on brain cell death (n=4 for each group; ANOVA followed by Bonferroni post-hoc tests for statistical analysis). Annexin-FITC and PI staining reveals presence of high numbers of apoptotic and necrotic cells in brain cortical region and PRL post-treatment markedly reduces the number of apoptotic and necrotic cells. (Obsevation under 40X magnification).

7.4. DISCUSSION

It is scientifically well established that the versatile peptide hormone PRL exerts pleiotropic effects in physiological systems including various activity in the central nervous system (CNS), especially, in the hippocampal region of brain [28]. The fact that neuroprotection in hippocampus of rat brain is observed during lactation [13, 11, 28], the role prolactin might play in the entire process has been widely investigated. It has been proved that PRL treatment reduces glutamate excitotoxicity induced elevated levels of intra-cellular calcium and apoptotic cell death in primary hippocampal cultures [14]. According to another study, neuronal PRL increased in juvenile rat brain as a result of hypoxia ischemic insult, though intracerebroventricular (i.c.v.) treatment with rat PRL (rPRL) failed to confer neuroprotection in the same model [29]. But, PRL reportedly protected hippocampal cells in chronic stress conditions and promoted neurogenesis in hippocampus [30]. The ability of peripheral PRL to

cross blood brain barrier via the PRL receptors (PRLR) [30] combined with its neuroprotective ability makes it an interesting candidate for future neurotherapeutics. Hence, this study tried to evaluate the therapeutic ability of PRL against global cerebral ischemia in rat model induced by BCCAO.

Elevated blood pressure, a common phenomenon in cerebral ischemia [22], was observed in the vehicle group animals. There was a 40% (approx.) increase in SBP of animals in vehicle group as compared to that of sham. However, treatment with all three doses of PRL showed significant restoration of SBP. Lowering of the elevated SBP by 22.72% in Treated-A group revealed a non-significant difference in SBP between Treated-A and sham group. In Treated-B and Treated-C group SBP is lowered by 15.15% and 17.42%, respectively, in comparison to Vehicle group. An increase in heart rate (~25%) of the vehicle group animals was observed as compared to that of the sham group. But, animals in Treated-A group showed restored heart rate. Treated-B group also showed significant restoration of heart rate (by ~14.8%) as compared to the vehicle group rats, but no significant change was observed in Treated-C group. Both Treated-A and Treated-B group of animals also demonstrated restored rCBF as compared to the lowered rCBF observed in vehicle group. Animals in Treated-A and Treated-B group had an increase of ~44.4% and ~38.33% in rCBF with respect to that of vehicle group animals, but Treated-C group did not show any significant change. Overall, the two highest doses of PRL (1mg/Kg and 0.5mg/Kg) were able to re-establish the physiological conditions of animals altered due to ischemic injury.

Ischemic injury leads to accumulation of excitatory amino acid glutamate in brain tissues [31] which further leads to loss of calcium homeostasis [32]. The increased calcium concentration leads to release of endogenous GABA molecules [33] leading to elevated levels of GABA in

various brain parts which remains unrestored even after 168 hrs of ischemia reperfusion (IR) injury [34]. Another molecule playing an important role in ischemic pathophysiology is nitric oxide which leads to increased infarct volume and BBB damage [35]. Due to highly volatile nature of NO, it is rapidly converted into nitrite and nitrate [36] and due to short half-life of nitrite, nitrate is measured for estimation of NO concentration. In this study, it was observed that animals in Treated-A and Treated-B group showed significant restoration of glutamate levels in cortical region ($p < 0.001$ and $p < 0.01$ respectively) and glutamate levels of cerebellar and hippocampal regions were restored only in Treated-A group ($p < 0.01$ and $p < 0.001$, respectively) as compared to vehicle group animals. Treated-C group did not show any significant restoration in glutamate levels in any of the cerebral regions in comparison to vehicle group animals. Significant decrease in GABA levels were observed in cortex and hippocampus of Treated-A group ($p < 0.001$) and also in cerebellum ($p < 0.05$) as compared to vehicle group animals. No significant changes in GABA levels were observed in any of the brain regions in either of Treated-B or Treated-C group of animals. Treatment with high doses of PRL (1mg/Kg and 0.5mg/Kg) significantly lowered the calcium concentration in cortex and hippocampus ($p < 0.001$) whereas Treated-C group had no significant effect on calcium levels was observed in these regions in as compared to vehicle group. Interestingly, in hippocampal region, all three doses of PRL significantly reduced the heightened calcium level ($p < 0.001$) with respect to that of vehicle group. This might be attributed to the ability of PRL to preserve calcium homeostasis during glutamate excitotoxicity in hippocampal neurons [14]. It was observed that though PRL induced a small increase in concentration of hippocampal calcium, but PRL pre-treatment significantly reduced the calcium levels in hippocampal neurons exposd to glutamate excitotoxicity [14]. The mechanism of PRL in restoring the calcium concentration induced due to glutamate excitotoxicity is not properly understood yet, but it has been reported

that PRL might combat the glutamate excitotoxicity by exerting an antagonistic effect on the glutamate receptors, thereby reducing calcium overload [14]. In the present study also, it is observed that PRL significantly restored calcium concentration in cortex, cerebellum and hippocampus regions. All three doses of PRL also significantly reduces nitrate concentration in cortex ($p < 0.001$), but in other two regions, i.e., cerebellum and hippocampus, only Treated-A group shows significant lowering of nitrate levels compared to vehicle group animals ($p < 0.01$). When compared to vehicle group, no significant changes in nitrate concentrations were observed in cerebellum and hippocampus regions of Treated-B and Treated-C group of animals. The evaluation of the neurotransmitter and biochemical parameters revealed that though the lower doses of PRL might not be very effective in reinstating the levels of these indispensable molecular mediators of ischemia, restoration of the levels of these molecules were achieved by administration of the highest dose (1mg/kg) of PRL.

Brain infarction is the hallmark of ischemic reperfusion injury and neuroprotection strategies aim at reducing the infarct volume [2]. This study revealed that though all three doses of PRL significantly reduce infarction volume in brain ($p < 0.001$) in comparison to infarct volumes observed in vehicle groups, Treated-A group had most successful effect on cerebral infarction reduction. Another characteristic of cerebral ischemia is increased brain water content [2] resulting due to BBB damage [37]. Compared to the vehicle group animals, where an increased brain edema by 23.8% was observed (compared to sham group), cerebral edema was significantly reduced in Treated-A and Treated-B group of animals ($p < 0.001$), but Treated-C group did not show any significant restoration. Decrease in cerebral infarct volume and cerebral edema by PRL treatment highlights the ability of PRL in ameliorating the ischemic damage incurred by brain tissues. Also, it was observed that number of apoptotic and necrotic

cells were markedly reduced in the Treated-A group, confirming the neuroprotective potential of this dose.

7.5. CONCLUSION

The present study was aimed at evaluating the therapeutic ability of PRL in cerebral IR injury. The results obtained strongly suggest that administration of PRL not only successfully restored the physiological and biochemical parameters, it also lowered the increased neurotransmitter levels which is a prominent outcome of ischemic insult. Also, PRL treatment significantly reduced tissue damage occurring due to IR injury, by lowering cerebral infarction and cerebral edema in rodent model. Though lower doses were unable to combat every aspect of global cerebral ischemia, the highest dose of PRL (1mg/Kg) significantly ameliorated the ischemic condition observed in brain. The study henceforth suggests that PRL might be a probable molecule which could be developed into future neurotherapeutics.

References

1. Jain KK. Neuroprotection in cerebrovascular disease. Expert. Opin. Investig. Drugs. 2000; 9(4):695-711. doi: 10.1517/13543784.9.4.695.
2. Xing C, Arai K, Lo EH, Hommel M. Pathophysiologic cascades in ischemic stroke. Int J Stroke. 2012; 7(5):378-85. doi: 10.1111/j.1747-4949.2012.00839.x.
3. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med. 2006; 3(11):e442. doi: 10.1371/journal.pmed.0030442.
4. Projections of mortality and burden of disease, World Health Organization. 2002–2030. http://www.who.int/healthinfo/global_burden_disease/projections2002/en/index.html.
5. Cheng YD, Al-Khoury L, Zivin JA. Neuroprotection for ischemic stroke: two decades of success and failure. NeuroRx. 2004; 1(1):36-45. doi: 10.1602/neurorx.1.1.36.
6. Brunton PJ, Russell JA. Endocrine induced changes in brain function during pregnancy. Brain Res. 2010; 1364:198-215. doi: 10.1016/j.brainres.2010.09.062.
7. Torner L, Toschi N, Pohlinger A, Landgraf R, Neumann ID. Anxiolytic and anti-stress effects of brain prolactin: improved efficacy of antisense targeting of the prolactin receptor by molecular modeling. J. Neurosci. 2001; 21(9):3207-14. doi: 10.1523/JNEUROSCI.21-09-03207.2001.
8. Torner L, Neumann ID. The brain prolactin system: involvement in stress response adaptations in lactation. Stress. 2002; 5(4):249-57. doi: 10.1080/1025389021000048638.
9. De Vito WJ, Stone S, Shamgochian M. Prolactin induced expression of glial fibrillary acidic protein and tumor necrosis factor-alpha at a wound site in the rat brain. Mol. Cell. Endocrinol. 1995; 108(1-2):125-30. doi: 10.1016/0303-7207(94)03465-6.
10. Walker TL, Vukovic J, Koudijs MM, Blackmore DG, Mackay EW, Sykes AM, Overall RW, Hamlin AS, Bartlett PF. Prolactin stimulates precursor cells in the adult mouse hippocampus. PLoS One. 2012; 7(9):e44371. doi: 10.1371/journal.pone.0044371.
11. Cabrera V, Cantú D, Ramos E, Vanoye-Carlo A, Cerbón M, Morales T. Lactation is a natural model of hippocampus neuroprotection against excitotoxicity. Neurosci. Lett. 2009; 461(2):136-9. doi: 10.1016/j.neulet.2009.06.017.
12. Vergara-Castañeda E, Grattan DR, Pasantes-Morales H, Pérez-Domínguez M, Cabrera-Reyes EA, Morales T, Cerbón M. Prolactin mediates neuroprotection against excitotoxicity in primary cell cultures of hippocampal neurons via its receptor. Brain. Res. 2016; 1636:193-9. doi: 10.1016/j.brainres.2016.02.011.
13. Vanoye-Carlo A, Morales T, Ramos E, Mendoza-Rodríguez A, Cerbón M. Neuroprotective effects of lactation against kainic acid treatment in the dorsal hippocampus of the rat. Horm. Behav. 2008; 53(1):112-23. doi: 10.1016/j.yhbeh.2007.09.004.
14. Rivero-Segura NA, Flores-Soto E, de la Cadena SG, Coronado-Mares I, Gomez-Verjan JC, Ferreira DG, Cabrera-Reyes EA, Lopes LV, Massieu L, Cerbón M. Prolactin-

induced neuroprotection against glutamate excitotoxicity is mediated by the reduction of [Ca2+] i overload and NF-κB activation. PloS One. 2017; 12(5):e0176910. doi: 10.1371/journal.pone.0176910.
15. Pourheydar B, Soleimani Asl S, Azimzadeh M, Rezaei Moghadam A, Marzban A, Mehdizadeh M. Neuroprotective effects of bone marrow mesenchymal stem cells on bilateral common carotid arteries occlusion model of cerebral ischemia in rat. Behav. Neurol. 2016. doi: 10.1155/2016/2964712.
16. Liu Z, Chen X, Gao Y, Sun S, Yang L, Yang Q, Bai F, Xiong L, Wang Q. Involvement of GluR2 up-regulation in neuroprotection by electroacupuncture pretreatment via cannabinoid CB1 receptor in mice. Sci. Rep. 2015; 5:9490. doi: 10.1038/srep09490.
17. Pujari RR, Vyawahare NS, Kagathara VG. Evaluation of antioxidant and neuroprotective effect of date palm (Phoenix dactylifera L.) against bilateral common carotid artery occlusion in rats. Indian J Exp Biol. 2011; 49.8: 627-633.
18. Wang X, Lin F, Gao Y, Lei H. Bilateral common carotid artery occlusion induced brain lesions in rats: A longitudinal diffusion tensor imaging study. 2015; 33(5):551-8. Magn. Reson. Imaging. doi: 10.1016/j.mri.2015.02.010.
19. Markus HS. Cerebral perfusion and stroke. J. Neurol. Neurosurg. Psychiatry. 2004; 75(3):353-61. doi: 10.1136/jnnp.2003.025825.
20. Li L, Ke Z, Tong KY, Ying M. Evaluation of cerebral blood flow changes in focal cerebral ischemia rats by using transcranial Doppler ultrasonography. Ultrasound in medicine & biology. 2010; 36(4):595-603. doi: 10.1016/j.ultrasmedbio.2010.01.005.
21. A Perez-Trepichio AD, Williams JL, Block CH, Jones SC. Cardiovascular changes during focal cerebral ischemia in rats. Stroke. 1993; 24(5):691-6.
22. Lattanzi S, Silvestrini M, Provinciali L. Elevated blood pressure in the acute phase of stroke and the role of Angiotensin receptor blockers. Int J Hypertens. 2013. doi: 10.1155/2013/941783.
23. Hutchinson PJ, O'Connell MT, Al-Rawi PG, Kett-White CR, Gupta AK, et al. Increases in GABA concentrations during cerebral ischaemia: a microdialysis study of extracellular amino acids. J. Neurol. Neurosurg. Psychiatry. 2002;72(1):99-105. doi: 10.1136/jnnp.72.1.99.
24. Phillis JW, Smith-Barbour M, Perkins LM, O'regan MH. Characterization of glutamate, aspartate, and GABA release from ischemic rat cerebral cortex. Brain Res. Bull. 1994; 34(5):457-66. doi: 10.1016/0361-9230(94)90019-1.
25. Dawson TM, Steiner JP, Dawson VL, Dinerman JL, Uhl GR, Snyder SH. Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. Proc. Natl. Acad. Sci. U.S.A. 1993;90(21):9808-12. doi: 10.1073/pnas.90.21.9808.
26. Fassbender K, Fatar M, Ragoschke A, Picard M, Bertsch T, Kuehl S, Hennerici M. Subacute but not acute generation of nitric oxide in focal cerebral ischemia. Stroke. 2000; 31(9):2208-11. doi: 10.1161/01.STR.31.9.2208.

27. Dostovic Z, Dostovic E, Smajlovic D, Ibrahimagic OC, Avdic L. Brain edema after ischaemic stroke. Arch. Med. Res. 2016; 70(5):339. PMID: 27994292.
28. Cabrera V, Ramos E, González- Arenas A, Cerbón M, Camacho- Arroyo I, Morales T. Lactation R educes Glial Activation Induced by Excitotoxicity in the Rat Hippocampus. J. Neuroendocrinol. 2013; 25(6):519-27. doi: 10.1111/jne.12028.
29. Torner L. Actions of prolactin in the brain: from physiological adaptations to stress and neurogenesis to psychopathology. Front. Endocrinol. 2016; 7:25. doi: 10.3389/fendo.2016.00025.
30. Möderscheim TA, Gorba T, Pathipati P, Kokay IC, Grattan DR, Williams CE, Scheepens A. Prolactin is involved in glial responses following a focal injury to the juvenile rat brain. Neurosc. 2007; 145(3):963-73. doi:10.1016/j.neuroscience.2006.12.053.
31. Torner L, Karg S, Blume A, Kandasamy M, Kuhn HG, Winkler J, Aigner L, Neumann ID. Prolactin prevents chronic stress-induced decrease of adult hippocampal neurogenesis and promotes neuronal fate. J. Neurosci. 2009; 29(6):1826-33. doi: 10.1523/JNEUROSCI.3178-08.2009.
32. Lai TW, Zhang S, Wang YT. Excitotoxicity and stroke: identifying novel targets for neuroprotection. Prog. Neurobiol. 2014; 115:157-88., Prog. Neurobiol. doi: 10.1016/j.pneurobio.2013.11.006.
33. Schwartz- Bloom RD, Sah R. γ- Aminobutyric acidA neurotransmission and cerebral ischemia. J. Neurochem. 2001; 77(2):353-71. doi: 10.1046/j.1471-4159.2001.00274.x.
34. Ramanathan M, Babu CS, Justin A, Shanthakumari S. Elucidation of neuroprotective role of endogenous GABA and energy metabolites in middle cerebral artery occluded model in rats. Indian. J. Exp. Biol. 2012; 50(6):391-7. PMID: 22734249.
35. Chen ZQ, Mou RT, Feng DX, Wang Z, Chen G. The role of nitric oxide in stroke. Med. Gas. Res. 2017; 7(3):194. doi: 10.4103/2045-9912.215750.
36. Rao AM, Dogan A, Hatcher JF, Dempsey RJ. Fluorometric assay of nitrite and nitrate in brain tissue after traumatic brain injury and cerebral ischemia. Brain Res. 1998; 793(1-2):265-70. doi: 10.1016/S0006-8993(98)00183-8.
37. Michinaga S, Koyama Y. Pathogenesis of brain edema and investigation into anti-edema drugs. Int. J. Mol. Sci. 2015; 16(5):9949-75. 16.5 (2015) 9949-75. doi: 10.3390/ijms16059949.