CHAPTER 6

IN-VIVO EVALUATION OF NEUROPROTECTIVE EFFICACY OF ESTETROL

Chapter Highlights

- ***Estetrol is a fetal hormone exerting neuroprotective effects in neonatal hypoxic–ischemic encephalopathy***
- ***Effect of Estetrol in ameliorating global cerebral ischemia is studied using BCCAO model***

ABSTRACT

Cerebral ischemia is a severe disease, causing deaths and disabilities worldwide and lack of proper therapeutic approaches makes it more deadly. Designing of novel therapeutics for combating ischemic brain damage is presently the need of the hour. This study focuses on evaluating the neuroprotective potential of the fetal estrogen, estetrol and establishes estetrol as a potent neuroprotectant which is capable of improving pathophysiological conditions associated with ischemic brain damage. Global cerebral ischemia was induced in adult mice by bilateral carotid artery occlusion followed by estetrol post treatment. Further, physiological, biochemical and histopathological studies were performed to evaluate efficacy of estetrol. The study reveals that estetrol post-treatment reduces cerebral infarction and brain swelling. Evan's Blue extravasation study shows that estetrol post-treatment restores integrity of blood-brain barrier. Also post-treatment with estetrol restores the level of calcium, nitrate and other neurotransmitters in different brain compartments, viz., cortex, hippocampus and cerebellum. Estetrol post-treatment also restores morphological and cellular damage observed in ischemic brain. Estetrol being a molecule produced by human fetal liver, is supposed to have very few

side effects if used as a therapeutic agent and its pharmacokinetic profiling in humans also showed that it was benign. The ability of estetrol to confer neuroprotection in ischemic insult along with beneficial effects on human physiological system, suggests that estetrol can be designated as a worthy candidate for future neurotherapeutics designing.

6.1. INTRODUCTION

Cerebral ischemia reperfusion injury (CIRI) has been connoted as one of the leading causes of death and disability globally [1]. The induction of cerebral ischemia occurs as a result of reduced cerebral blood flow (CBF) [2,3] which consequently leads to lack of oxygen and nutrient supply to brain [4]. As a consequence of reduced oxygen supply, ATP reservoir is depleted inflicting severe neuronal damage [5]. Loss of ATP reservoir results in excessive release of the neurotransmitter glutamate, which further promotes major calcium influx [5]. Excessive calcium accumulation causes generation of free radicals like superoxide ion, hydroxyl ions and hydrogen peroxide causing mitochondrial dysfunction further leading to formation of nitric oxide (NO), which is another source for free ionic radical production [6]. Free radical generation causes disruption of blood brain barrier (BBB) [7] and also causes swelling of brain tissue, otherwise known as cerebral edema [8].

Though the treatment of cerebral ischemic pathophysiology relies on providing adequate blood supply to the oxygen deprived brain region [9], it has been reported that reperfusion followed by ischemic insult further leads to damage of brain tissues [9,10]. This phenomenon further causes oxidative stress in neuronal cells and increases vulnerability of brain towards ischemic damage [11]. Presently, recombinant tissue plasminogen activator (r-tPA) remains the only therapeutic agent for combating CIRI [5]. But, the limitations like limited window period and high chances of resulting thrombolysis [5] necessitates designing of alternative therapeutics

for treating cerebral ischemia. In order to investigate mechanisms involved in cerebral ischemia and to develop novel therapeutics to combat the pathophysiology of CIRI, a reproducible model is absolutely necessary. Bilateral common carotid artery occlusion (BCCAO) is a simple, reproducible and reliable method which is frequently used to induce global cerebral ischemia in rat and mice model [12]. The model is widely accepted for evaluating drug efficacy in cerebral ischemia and to study the relevant physiological and biochemical changes associated with ischemic pathophysiology [13-15].

Estetrol (E4) is major Estradiol (E2) metabolite which is exclusively synthesized by fetal liver during human pregnancy [16]. Recent scientific findings suggest that E4 can be potentially used for hormone therapy (HT), contraception, preventing menopausal hot flushes and osteoporosis in postmenopausal women [17-19]. Also, pharmacokinetic study conducted in postmenopausal human females revealed that it is safe and tolerable exhibits bioavailability proportional to oral dose [18]. Recently, neuroprotective effects of E4 has been reported in animal model of neonatal hypoxic–ischemic encephalopathy (HIE) [16]. E4 has been reported to display strong anti-oxidant activity [16, 20] and to manifest neuroprotection in hippocampus and cortex region of brain in HIE induced rat pup [16]. The ability of E4 as a competent anti-oxidant and its neuroprotective ability suggests that E4 might be able to combat to pathophysiology of CIRI. Therefore, in this study, we have tried to evaluate the efficacy of E4 in ameliorating cerebral ischemia induced by BCCAO in mice model.

6.2. MATERIALS AND METHODS

All protocols animal handling and experiments were approved by Central Animal Ethical Committee at Institute of Medical Sciences, Banaras Hindu University, Varanasi (Registration No. 542/02/ab/CPCSEA). Inbred male albino mice of weight 30-32 g were acclimatized for

two weeks under controlled temperature of 25±2 °C and constant humidity. A 12-h light/dark cycle was maintained and standard laboratory food and water was made freely accessible.

6.2.1. Induction of Global Cerebral Ischemia

The animals were fasted overnight with free access to water before performing surgical procedures. Global cerebral ischemia was induced in mice by performing BCCAO [12,14,15] after anesthetizing the animals with an intra-peritoneal injection of a combination of ketamine (50 mg/kg b.w.) and xylazine (10 mg/kg b.w.). The anesthetized mice were fixed on surgical table in supine position and the skin of the neck region was sterilized using 75% Ethanol and Povidone iodine solution after shaving off the fur covering the area. Both the common carotid arteries (CCAs) were exposed by a vertical midline neck incision and were loosely encircled with loose aseptic non-absorbable silk sutures (3-0). The suture around both left and right carotid artery was tightened strongly after placing a small piece of saline soaked cotton in between each of the CCAs and the suture. The vessels were occluded for 30 minutes, followed by reperfusion of 6 hours. Reperfusion was performed by careful cutting of the silk suture loop with a sharp surgical blade and removal of both the cotton pieces and sutures. The incision was stitched with the aseptic 3-0 silk sutures and Povidone iodine ointment was applied on the wound area. In sham group, same surgical procedures were performed except occlusion of the CCAs. Body temperature of all the animals were maintained at 37±0.5°C throughout the surgery and was monitored via a rectal thermometer. After the surgery the animals were placed in their cages under controlled room temperature of 25±2°C and food and water was made available *ad-libitum*.

6.2.2. Preparation of E4 solutions and Dosage

To evaluate the neuroprotective effect of E4, three different doses (5 mg/kg, 2.5 mg/kg and 1 mg/kg) were prepared. The doses were decided as per the study on neuroprotective effect of E4 in nHIE reported by Tskitishvili et al [16]. For further reference, 5mg/kg is referred to as Dose1, 2.5mg/kg as Dose2 and 1mg/kg as Dose3. Different concentrations of E4 (Sigma-Aldrich; Cat No. : SML 1523) solution was prepared in normal saline and an equal volume of 5µl/g was administered intra-peritoneally (I.P.) to the mice [16].

For the experimental studies, 75 adult male mice were divided into five groups for each parameter, i.e., sham (n=5), vehicle (n=5), for each dose of E4 treated (n=5). All the animals in E4 treated groups (all three doses) were injected E4 (according to the groups) 30 minutes after ischemia induction by occlusion. The animals in sham group neither received any drug nor vehicle. The experimental work-flow is given in Fig. 6.1.

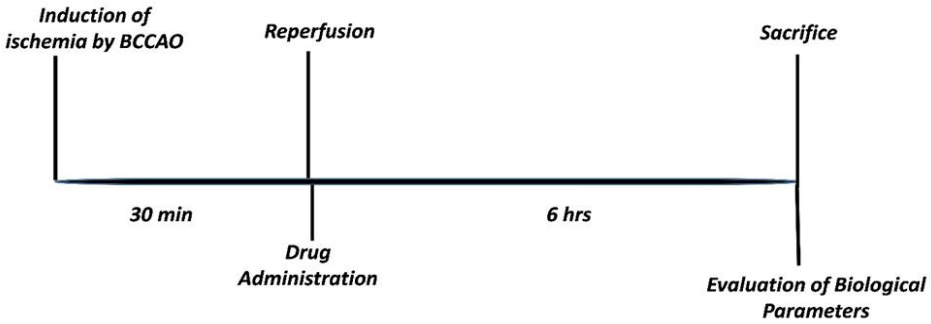

Fig. 6.1.: Schematic representation of experimental procedure for *in-vivo* studies.

6.2.3. Cerebral Infarction Determination

To evaluate the efficacy of various doses of E4 on cerebral infarction, triphenyltetrazolium chloride (TTC) staining of mice brain was performed (n=6 for each group) [21]. E4 post-treatment (Dose1, Dose 2 and Dose 3) was done in ischemia induced mice and vehicle group

mice by above mentioned method. The mice were anesthetized by chloroform and sacrificed by cervical dislocation after 6 hours of reperfusion. The sham group animals did not receive any vehicle or E4 post-treatment. The brains were incubated in -20°C for 5 minutes after removal from skull and immediate rinsing in normal saline, following which TTC staining and calculation of infarction area was performed as per the protocol mentioned in Section 4.2.6.

6.2.4. Blood Brain Barrier (BBB) Disruption

BBB disruption is associated with the onset on ischemic insult and extravasation of exogenous tracer dyes, such as Evan's Blue (EB), is a popular method of quantifying BBB permeability [20]. In this study (n=5 for each group), Evan's Blue (TCI Chemicals; Cat. No.: E0197) extravasation method was used as described by Martin *et. al.*, 2010 [23] with slight modification, to investigate the effect of the three different doses of E4 (Dose1, Dose2 and Dose 3) on BBB permeability. Administration of EB and measurement of EB concentration was done as per section 4.2.7.

6.2.5. Cerebral Edema

Cerebral edema is one of the major consequences of cerebral ischemia due to arterial occlusion [24]. In the present study, cerebral edema was assessed by measuring the percentage of brain water content of the different groups (n=5 in each group). The brain water content percentage was calculated by the wet and dry-weight method [25]. The study was performed as per the protocol mentioned in section 4.2.8. The following formula was used for calculating the % brain water content:

$$\%\ of\ brain\ water\ content = 100 \times (wet\ weight - dry\ weight) \div dry\ weight$$

6.2.6. Determination of Glutamate and ɣ-Aminobutyric acid (GABA) concentration

CIRI is characterized by release of gl utamate, which is a key excitotoxic neurotransmitter [5]. Release of inhibitory amino acid GABA has also been associated with cerebral ischemia in various scientific studies [26, 27]. Glutamate and GABA concentrations are estimated in cortex, cerebellum and hippocampi of animals in each of the groups (n=5) by using the protocol as depicted in section 4.2.9.

6.2.7. Calcium Concentration Estimation

Binding of glutamate to N-Methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors promotes major calcium influx, which further causes degradation of essential membrane proteins [5]. Calcium concentration in different brain tissues (cortex, cerebellum and hippocampi) for each group (n=5) were determined using calcium detection kit (ab102505; Abcam) according to the protocol described in section 4.2.10.

6.2.8. Nitrate Estimation

Generation of nitric oxide (NO) is a marker of CIRI [28,29] and nitrate concentrations can be determined to estimate a change in NO concentration, since it is an unstable and rapidly decaying compound [30,31]. Nitrate levels in various brain parts such as cortex, cerebellum and hippocampi for all the each of the five groups (n=5) were measured as per section 4.2.11.

6.2.9. CBF Analysis

Restoration of cerebral blood flow (CBF) is the major goal to achieve while combating cerebral ischemia [5]. Regional cerebral blood flow (rCBF) of both left and right hemisphere of mice was measured using laser doppler blood flow (LDF) meter (INL191 Blood Flow Meter AD

Instruments Pty Ltd, Australia) after fixing the anesthetized animals on stereotaxic instrument (INCO, Ambala India). Briefly, the soft tissue from the skull surface was gently removed with sterile cotton swabs after exposing and disinfecting the skull bone with povidone-iodine. Using a low speed dental drill, a small area at 2 mm posterior and 3 mm lateral to the bregma of both the hemispheres were thinned [32] and LDF needle probe was fixed to obtain the cerebral blood flow. CBF was measured to monitor the induction of occlusion by ligation of CCAs, which showed that CBF is decreased by at least 80% during BCCAO. CBF was also recorded after 6hr of reperfusion for all the groups (n=5).

6.2.10. Histopathological study

Morphological changes in brain cortical region was evaluated by staining with Hematoxylin and Eosin (H&E). H&E staining of the brain tissues for each group (n=4) were performed as per the protocol described in 4.2.12.

6.2.11. Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests. A value of $p < 0.05$ was considered statistically significant. All data are expressed in mean ± S.D.

6.3. RESULTS

6.3.1. Dose dependent effect of E4 on cerebral infarction

The brain coronal sections of animals with CIRI (30 min ischemia/6 hrs reperfusion, i.e., 30/6 min /hr I/R) exhibited marked infarction area in the cortical regions (Fig. 6.2.). It was observed that all three doses of E4 reduced the cerebral infarction, but the animals receiving the Dose1

of E4 showed lowest percentage of cerebral infarction. As compared to 29.79% infarction showed by vehicle group animals, the animals in Dose1, Dose2 and Dose3 groups displayed 12.42, 15.77 and 19.31% of cerebral infarction, respectively. All the three doses significantly reduced the cerebral infarction as compared to that of vehicle group ($p < 0.001$).

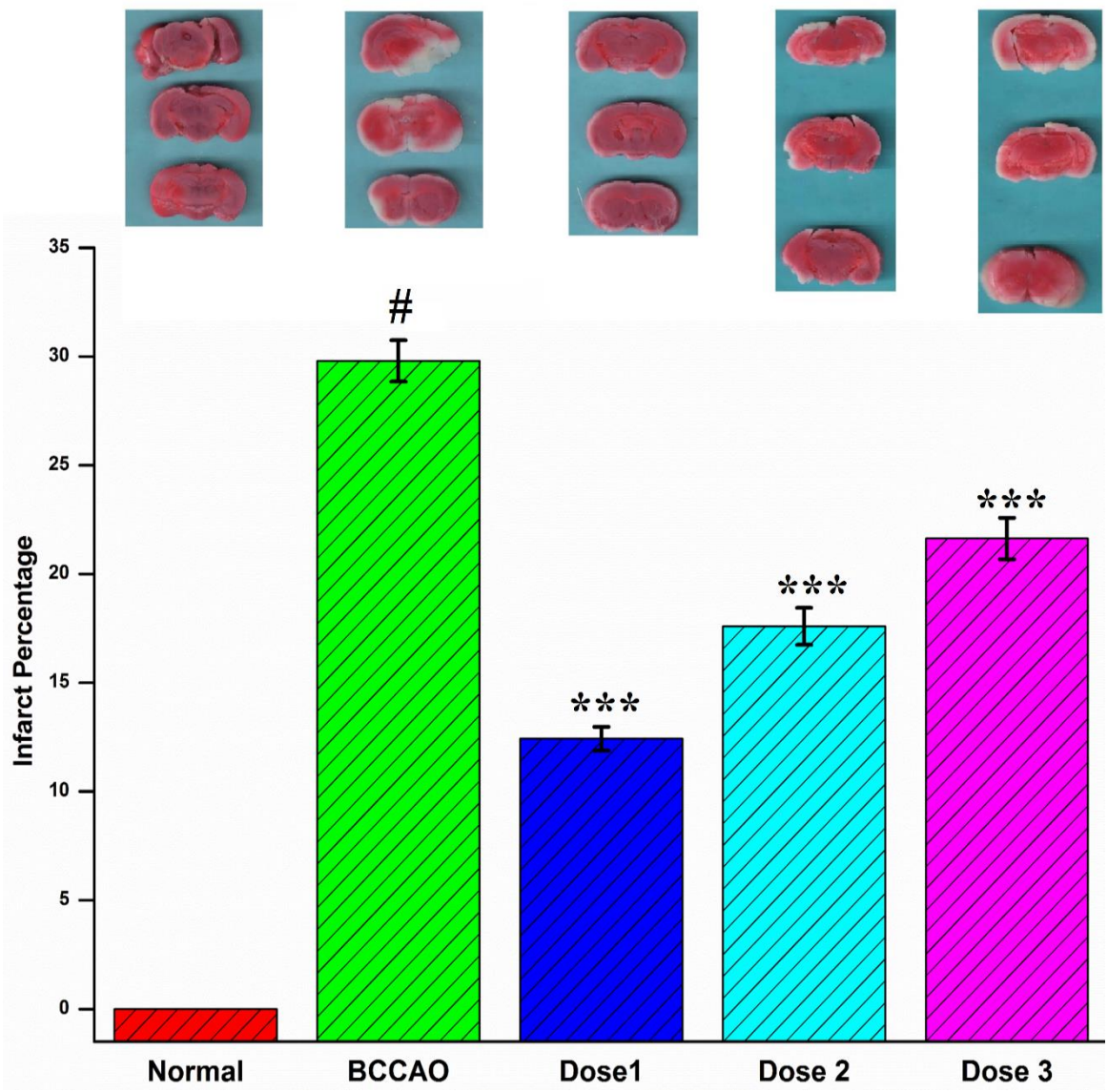

Fig. 6.2.: Effect of different doses of E4 on cerebral infarction. Post-treatment with Dose1 (5 mg/kg), Dose2 (2.5 mg/kg) and Dose3 (1 mg/kg) significantly reduces the cerebral infarction observed in vehicle group animals after 30 mins/6hr ischemia/reperfusion (I/R) injury. (n=6; ANOVA followed by Bonferroni 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).**

6.3.2. Restoration of BBB with E4 post-treatment

BBB disruption was estimated by calculating leakage of Evan's Blue in mice brain (Fig.6.3.). Compared to the animals in vehicle group, Evan's Blue extravasation in animals of Dose1 group was reduced by 91.96%. Concentration of Evan's Blue in animal brains of Dose2 and Dose3 groups were also reduced by 69.8 and 29.22 % respectively. Reduction of Evan's Blue extravasation by three doses of E4 was significant as compared to animals of vehicle group ($p < 0.001$).

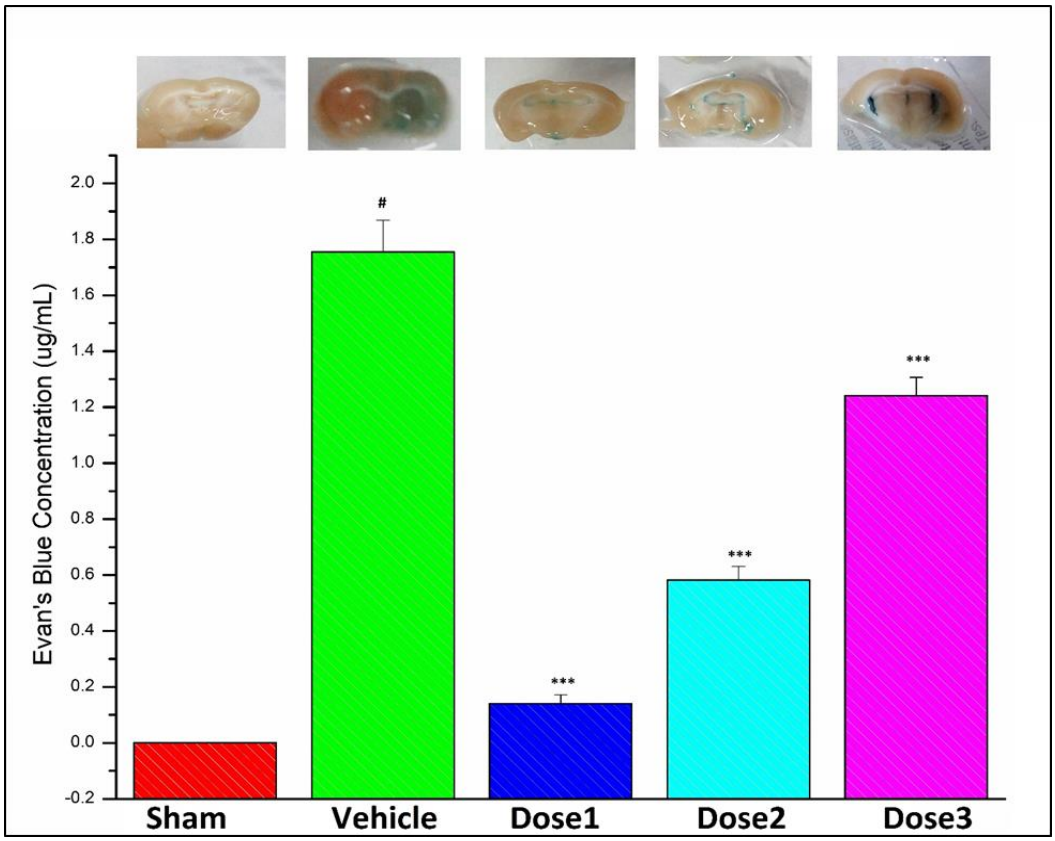

Fig. 6.3.: Effect of E4 post-treatment on EB extravasation in mice brain. Blue regions of brain signify EB accumulation is highest in the vehicle group animals (saline treated after 30 mins/6hr I/R injury). Among E4 treated groups, animals in Dose1 group (5 mg/kg) had least EB accumulation whereas those in Dose3 (1 mg/kg) group showed highest accumulation of EB. Animals in Dose2 groups (2.5 mg/kg) also showed significantly lower EB leakage as compared to vehicle group. (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).**

6.3.3. Post-treatment of E4 reduces brain swelling

Neuronal cell damage leads to accumulation of excess water in both intra and extracellular brain spaces leading to cerebral edema [8]. As compared to vehicle group, Dose1 and Dose2 post-treatment significantly reduced brain swelling and nearly restored the brain water level ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 6.4.). A non-significant decrease in brain edema percentage was observed in animals of Dose3 group as compared to animals in vehicle group. Also, percentage of brain edema of Dose1 and Dose3 groups were non-significant as compared to that of vehicle group animals.

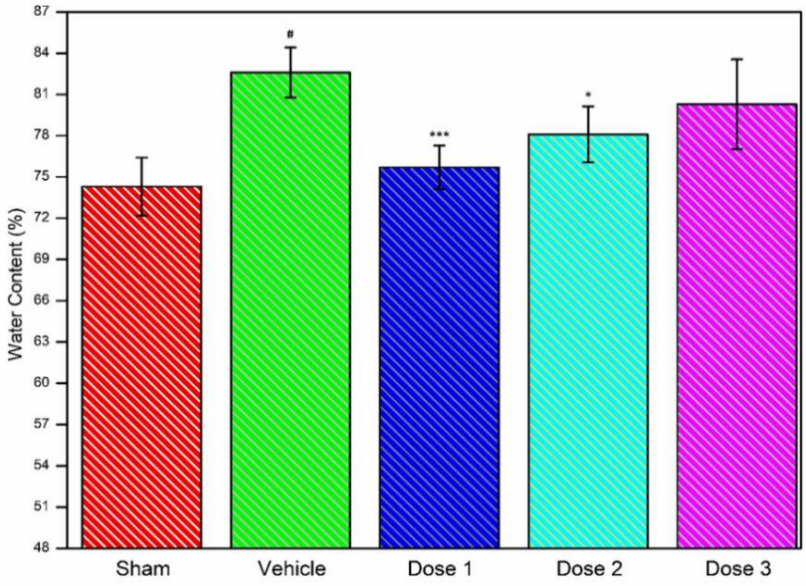

Fig. 6.4.: Effect of E4 post-treatment on brain-water content. Increased brain-water content is observed in vehicle group animals after 30 mins/6hr I/R injury. Dose1 (5 mg/kg) and Dose2 (2.5 mg/kg) of E4 significantly reduces brain water content. Dose3 (1 mg/kg) shows no significant effect on brain water content reduction. (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis; * signifies $p < 0.001$, * signifies $p < 0.05$ compared to vehicle group; # signifies $p < 0.001$ of vehicle group as compared to sham).**

6.3.4. Effect of E4 post-treatment on glutamate concentration

In animals of Dose1 and Dose2 groups, glutamate concentration in cortex was significantly reduced ($p < 0.001$ and $p < 0.01$, respectively) as compared to animals of vehicle group (Fig. 6.5.). No significant difference was observed in glutamate concentration of Dose3 and vehicle

group animals and also in that of Dose1 and Dose2 groups (Fig. 6.5.). Dose1 of E4 also significantly reduced glutamate concentration in cerebellum and hippocampus of mice brain ($p < 0.001$ and $p < 0.01$, respectively). Post-treatment with Dose2 also showed significant reduction of glutamate concentration in both cerebellum and hippocampus ($p < 0.05$) compared to vehicle group. Although, Dose3 reduced glutamate concentration in cerebellum significantly ($p < 0.05$), there was no significant difference in glutamate concentration in hippocampus of animals of vehicle and Dose3 groups. No significant difference was observed in hippocampal glutamate concentration in Dose1 and Dose2 groups of animals.

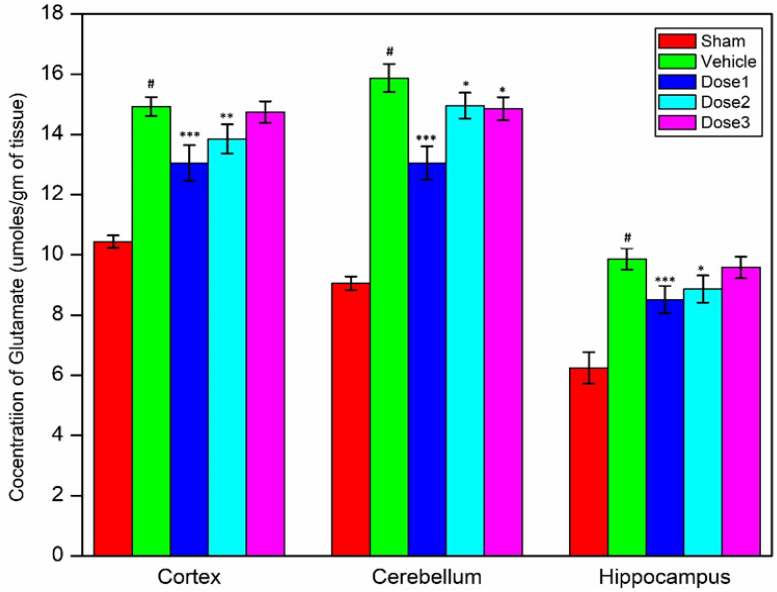

Fig. 6.5.: Effect of E4 post-treatment on glutamate concentration in various brain parts. Increased glutamate concentration in all brain regions is observed in vehicle group due to 30 mins/6hr I/R injury. Dose1 (5 mg/kg) and Dose 2 (2.5 mg/kg) significantly lower glutamate concentration in cortex, hippocampus and cerebellum. Dose 3 (1 mg/kg) shows significant reduction of glutamate concentration only in cerebellar region. (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis; * signifies $p < 0.001$, * signifies $p < 0.05$ compared to vehicle group; # signifies $p < 0.001$ of vehicle group as compared to sham).**

6.3.5. E4 post-treatment reduces GABA concentration in brain compartments

Post-treatment with Dose1 of E4 significantly lowered ($p < 0.001$) the elevated GABA concentration in all three brain parts, viz., cortex, cerebellum and hippocampus (Fig. 6.6.). In Dose2 pre-treated group also, a significant reduction of GABA level was observed in cortex,

hippocampus ($p < 0.01$) and cerebellum ($p < 0.05$) [Fig. 6.6.]. Dose3 post-treatment did not manifest any significant reduction in GABA levels in any of the brain compartments. In hippocampus, no significant difference was observed in animals of Dose1 and Dose2 groups.

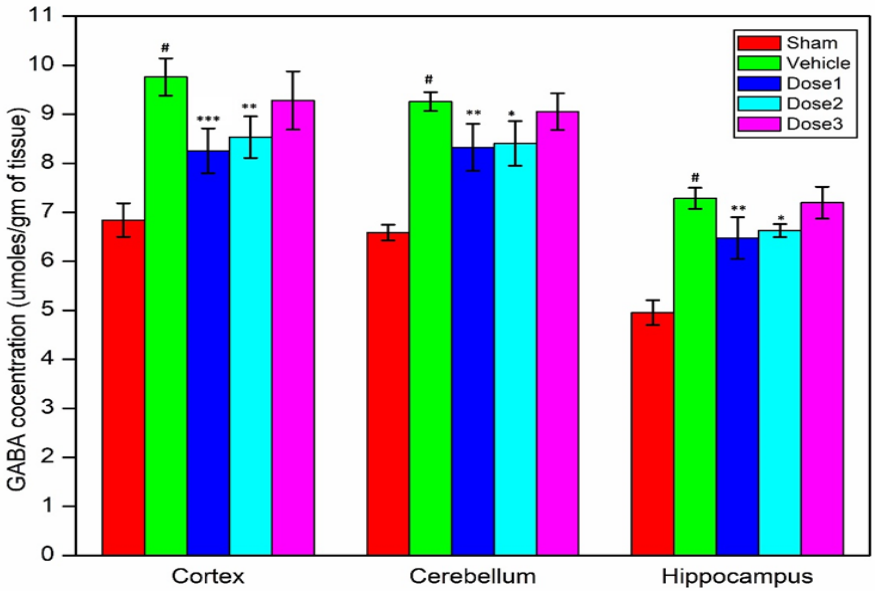

Fig. 6.6.: Effect of different doses of E4 on GABA levels in cortex, cerebellum and hippocampus. Increased GABA levels observed in vehicle group animals after 30 mins/6hr I/R injury. Dose1 (5 mg/kg) and Dose2 (2.5 mg/kg) significantly reduces GABA concentration in all three regions. Dose3 (1 mg/kg) shows no significant effect in any region. (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).**

6.3.6. Effect of E4 concentrations on cerebral calcium level

Post-treatment with Dose1 and Dose2 of E4 led to a significant reduction ($p < 0.001$) in calcium levels in cortex, cerebellum and hippocampus of mice brain as compared to vehicle group (Fig. 6.7.). Though Dose3 significantly reduced calcium level in cortex ($p < 0.05$) and cerebellum ($p < 0.001$), its effect on elevated calcium level in hippocampus was non-significant as compared to vehicle group. Also, there was no significant difference in hippocampal calcium level in Dose1 and Dose2 animal groups.

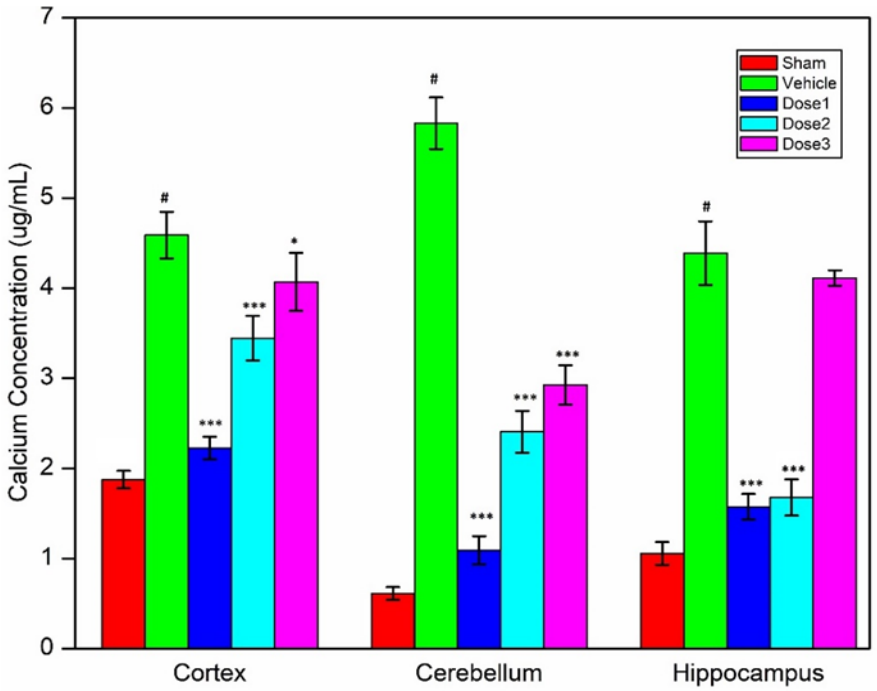

Fig. 6.7.: Effect of different doses of E4 on cerebral calcium levels. Increased concentration of calcium is observed in all three regions in vehicle group after 30 mins/6hr I/R injury. Restored calcium levels observed in cortex and cerebellum in Dose1 (5 mg/kg) and Dose2 (2.5 mg/kg) post-treated groups. Dose3 (1 mg/kg) has no significant effect on any region except cerebellum. (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis; * signifies $p < 0.001$, * signifies $p < 0.05$ compared to vehicle group; # signifies $p < 0.001$ of vehicle group as compared to sham).**

6.3.7. E4 attenuates nitrate level in brain

Nitrate levels in all three brain parts, i.e., cortex, cerebellum and hippocampus were reduced significantly ($p < 0.001$) by Dose1 post-treatment (Fig. 6.8.). In hippocampus, there was no significant effect of Dose2 and Dose3 on nitrate levels, whereas these two doses significantly lowered ($p < 0.001$) cerebellar nitrate levels. In cortex, Dose3 did not restore nitrate levels significantly as compared to vehicle group and also, there was no significant difference between nitrate levels of Dose1 and Dose2 groups of animals.

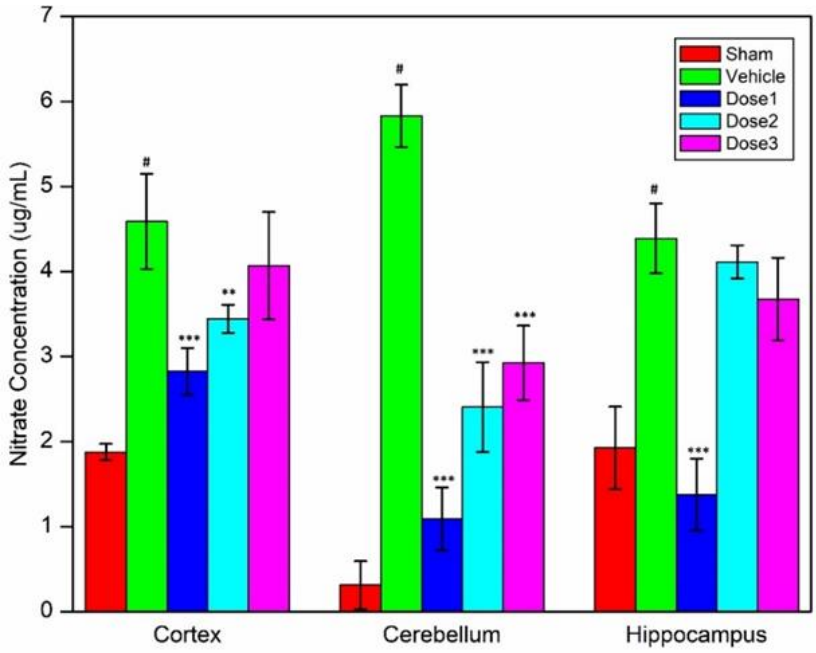

Fig. 6.8.: Effect of E4 post-treatment on brain nitrate concentration. Vehicle group shows increased cerebral nitrate concentration cortex, cerebellum and hippocampus due to 30 mins/6hr I/R injury. Restoration of nitrate concentration observed in all brain regions in Dose1 (5 mg/kg). Dose 2 (2.5 mg/kg) restores calcium level in cortex and cerebellum and Dose3 (1 mg/kg) restores calcium concentration in cerebellum only. (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).**

6.3.8. E4 partially restores CBF

Cerebral blood flow in both right and left hemispheres of mice at 6 h of reperfusion was measured and is represented in Fig.9. As compared to the sham group, CBF of vehicle group was lowered by 64.6%. Dose1 of E4 significantly increased ($p < 0.001$) CBF as compared to that of vehicle group, but Dose2 and Dose3 did not have any significant effect on CBF (Fig. 6.9.).

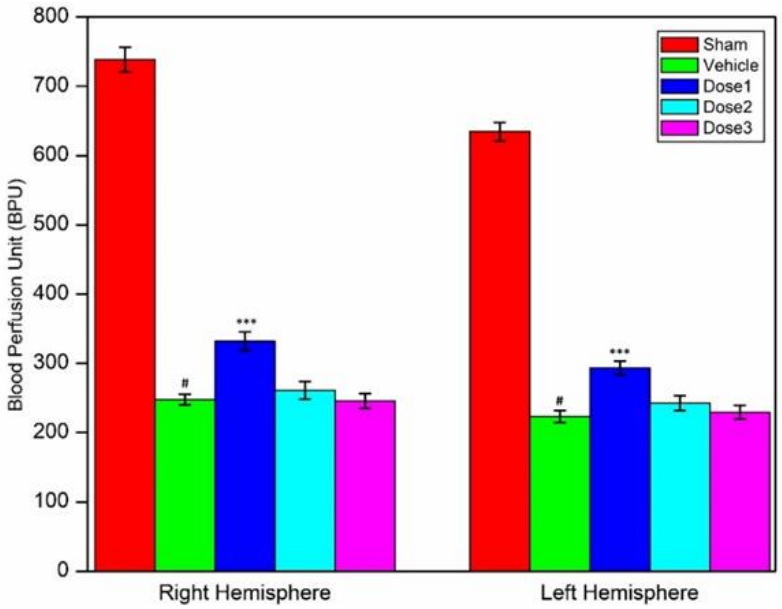

Fig. 6.9.: Effect of E4 post-treatment on CBF. Reduced CBF observed in both hemispheres in vehicle groups after 30 mins/6hr I/R injury. Dose1 (5 mg/kg) of E4 partially restores cerebral blood flow in both hemispheres. Dose2 (2.5 mg/kg) and Dose3 (1 mg/kg) have no significant effect on CBF in either hemisphere. (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).**

6.3.9. Estetrol treatment ameliorates morphological changes in brain cortical region

To evaluate the morphological changes in brain cortical region due to ischemia reperfusion injury and effect of E4 treatment, H&E stains were used. H&E staining revealed that higher number of vacuoles, small nuclei and deformed cytoblasts were present in the infarction area in vehicle group as compared to the treated (5mg/kg) group (Fig. 6.10.). The animals treated with 2.5 mg/kg E4 also showed reduced vacoulation whereas cortical region of 1mg/kg E4 treated animals was characterized by large number of vacuoles (Fig. 6.10.).

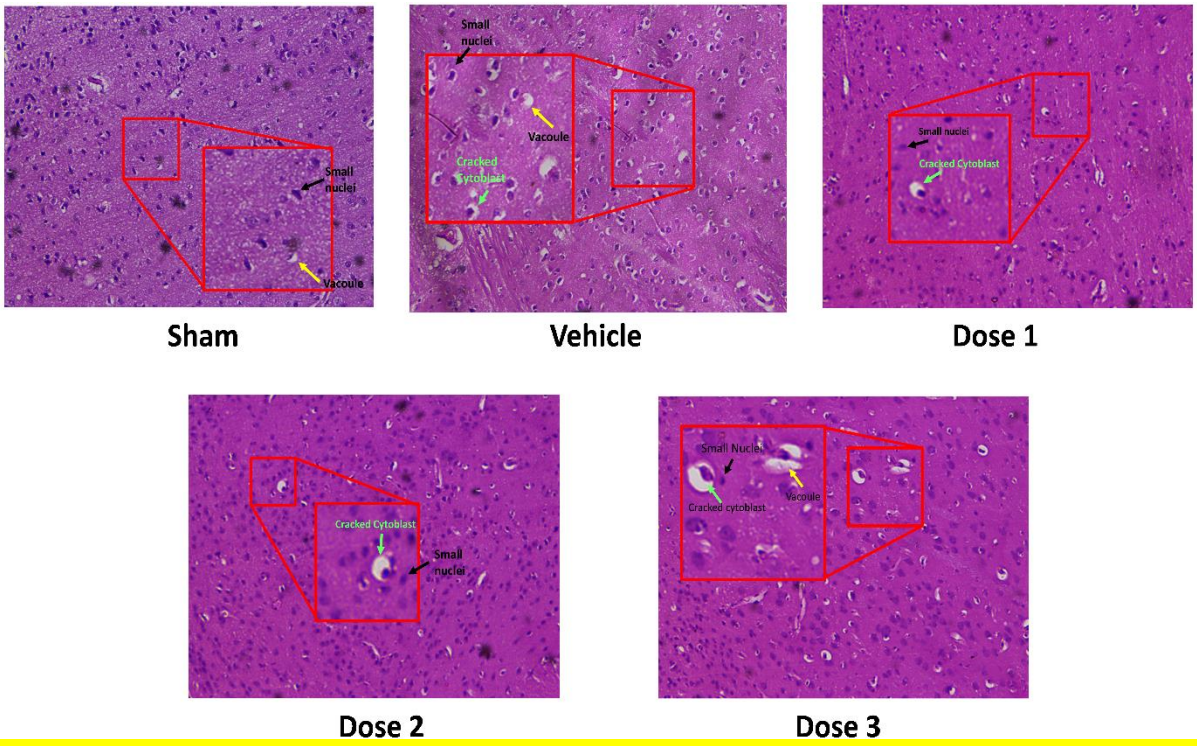

Fig. 6.10.: Hematoxylin and Eosin staining of the brain cortical region of different experimental groups (n=4 for each experimental group) reveals extensive tissue damage in the vehicle group characterized by the presence of numerous vacuoles (yellow arrows), cracked cytoblasts (green arrows) and small nuclei (black arrows). Less number of vacuoles are observed in Dose1 (5 mg/kg) and Dose2 (2.5 mg/kg) groups. A large number of vacuoles are observed in the Dose3 (1 mg/kg) group.

6.4. DISCUSSION

Previously considered as a weak estrogen [19], estetrol or E4 have been recently reported to administer neuroprotective effect in neonatal hypoxic–ischemic encephalopathy (HIE) [16]. E4 synthesis by fetal liver occurs exclusively during human pregnancy and differs from the other estrogenic molecules in the number of the free phenolic hydroxyl (OH) group [19]. According to recent studies, the neuroprotective ability of Estrogens strongly depends on the number of free OH group [33], which equips these molecules to combat the oxidative stress generated during neuronal disorders and neurodegeneration [16]. Possession of highest number

of free phenolic groups is suggestive of E4's strong anti-oxidative nature [16, 20], which might contribute to its ability to confer neuroprotection. Also, previous scientific studies regarding pharmaceutical properties of E4 reveals that due to its high water-solubility and an octanol–water parturition coefficient (Pow) of nearly 1.5, it is very likely to cross BBB (optimal Pow for passage through the BBB is 2) and manifest its effect in central nervous system (CNS) [19]. Affinity of E4 towards Estrogen receptors α and β (ERα and ERβ) and its ERα mediated neuroprotective action observed in HIE [16], are also indicative of its ability to offer neuroprotection in cerebral ischemia, since ERα is extremely necessary for estrogens to exhibit their neuroprotective ability during ischemic conditions [16]. In the present study, we for the first time evaluate the ability of E4 to combat global cerebral ischemia.

An onset of cerebral ischemic insult was confirmed by the presence of infarction regions in the brain tissue of the vehicle group animals. The results demonstrate that post-treatment with Dose1 of E4 (5mg/kg) reduced the cerebral infarction area by 58.29% and the other two doses Dose 2 (2.5 mg/kg) and Dose3 (1mg/kg) also showed a reduction of 47.03% and 35.15% respectively as compared to vehicle group. Reduced cerebral infarction in E4 treated groups indicates towards E4 neuroprotective ability in cerebral ischemia. A loss in BBB integrity is associated with cerebral ischemic pathophysiology [34, 35] and measurement of Evan's Blue extravasation effectively quantifies the severity of BBB breakdown. It was observed in the present study that Dose1, Dose2 and Dose3 significantly decreases Evan's Blue extravasation in mice brain as compared to vehicle group by 91.96, 69.8 and 29.22 % respectively. Post-treatment with Dose1 and Dose2 of E4 caused significant reduction of cerebral edema, which is another important marker of cerebral ischemia. Dose3 post-treatment did not show any significant effect on restoring brain water content. Ischemic conditions lead to increase in

concentration of excitatory amino acid glutamate [36], which plays a further role in neurodegeneration. To antagonize the effect of the excitatory amino acid glutamate, the inhibitory amino acid GABA is excessively released as a defense mechanism [37]. It was observed in the present study, that Dose1 and Dose2 significantly reduces the levels of Glutamate and GABA in cortex, cerebellum and hippocampus. Though Dose3 has a significant effect on reduction of Glutamate levels in cerebellum, it is unable to assert any significant lowering of glutamate in cortex and hippocampus or on GABA levels in any of the three brain compartments. No significant difference was observed in glutamate levels of Dose1 and Dose2 group animals in cortex and hippocampus and GABA levels in hippocampus. Excessive glutamate release further causes NMDAR activation and thus leads to major calcium influx [38] which in turn leads to nNOS activation [29], thus triggering generation of Nitric oxide [NO] [5]. NO is a highly volatile molecule and is converted rapidly into nitrite and nitrate. Nitrite also has a very short half-life. Hence, nitrate concentration in various brain regions were measured as an indicative of total NO concentration. It was observed that Dose1 significantly restores calcium and nitrate levels in all cortex, cerebellum and hippocampus, whereas Dose2 did not have any significant effect on hippocampal nitrate levels. But, Dose2 significantly lowered calcium level in all three brain compartments and reduced nitrate concentration in cortex and cerebellum significantly. Dose3 did not have any significant effect on hippocampal nitrate and calcium concentration, but reduced calcium level significantly in cortex and cerebellum. Dose3 was also non-significant in reducing nitrate levels in cortex region. These experimental data strongly suggest that E4 successfully combats pathophysiological changes induced by global cerebral ischemia by reducing the BBB damage, neurotransmitter levels and calcium and nitrate concentrations. Effect of E4 on CBF was also studied and it was found that

Dose1 had a significant effect in increasing CBF in both right- and left-brain hemispheres. Dose2 and Dose3 did not have any significant effect on CBF restoration.

Glutamate excitotoxicity plays a pivotal role in cerebral ischemia by steering calcium influx, which in turn induces oxidative and nitrosative stress [6]. Conferring neuroprotection by lowering the glutamate load is a well-established feature of the estrogens' neuroprotective mechanism [16, 39]. Though not properly understood yet, it is possible that E4 being an estrogen, undertakes a similar mechanism, because the present study revealed that E4 post-treatment (highest dose of 5 mg/kg) reduces glutamate concentration in all three cerebral compartments examined in the present study. It is expected that with lowering of glutamate excitotoxicity, the concentration of the other neurotransmitters like calcium, nitrate and GABA will also be restored. The concentrations of these neurotransmitters were evaluated in different brain regions in order to understand effect of E4 in different parts of brain. It was observed that the reduction of glutamate excitotoxicity has a simultaneous effect on lowering the calcium levels and subsequent oxidative and nitrosative damage. Glutamate excitotoxicity reportedly causes an increase in the cerebral GABA concentration [40] and the reduction of GABA levels in E4 post-treated groups signifies that E4 is able to ameliorate glutamate excitotoxicity. Glutamate excitotoxicity is also linked with cerebral infarction and BBB damage, which leads to brain edema. The restoration of the neurotransmitter concentrations in different brain parts and the previously mentioned cerebral parameters, indicates that the neuroprotective ability of E4 might be mediated by its ability to ameliorate ischemia-induced glutamate excitotoxicity in the brain.

The present study suggests that though the lowest dose of E4 (1mg/kg) did not manifest strong combating ability towards CIRI induced brain damage, higher doses (5mg/kg and 2.5 mg/kg)

were effective against global cerebral ischemia. Specifically, Dose1 (5mg/kg) effectively and significantly ameliorated the ischemic pathophysiological conditions which is in accordance to a previous scientific study reporting 5mg/kg dose of E4 as neuroprotective in HIE [16].

6.5. CONCLUSION

The recent study for the first-time reports E4's neuroprotective ability in CIRI in *in-vivo* mice model. Post-treatment by E4 restores BBB integrity and brain water content, reduces the neurotransmitters and biochemical markers like calcium and nitrate levels in cortex, hippocampus and cerebellum in mice brain. E4 being a natural fetal hormone could be a safe option for neurotherapeutics in human and its highly selective nature poses lesser risks of a effects [16]. Unlike Estradiol (E2), which is a well-established neuroprotectant for ischemic stroke, E4 is not inactivated in human circulation due to binding of sex hormone binding globulin (SHBG) and albumin [18], so it is readily bio-available [18]. The fact that E4 is safe and well-tolerated in human and possess high biological potency pertaining to its long half-life [18] makes it an efficacious and potent molecule for combating cerebral ischemia. The neuroprotective ability of E4 certainly establishes it as a future candidate for development of neurotherapeutics.

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