CHAPTER 8

NEUROPROTECTIVE EFFECT OF CHLOROGENIC ACID IN GLOBAL CEREBRAL ISCHEMIA-REPERFUSION RAT MODEL

Highlights of the chapter

- *The neuroprotective effect of chlorogenic acid (CGA) was evaluated in bilateral common carotid artery occlusion-reperfusion induced cerebral ischemia in rats after administration of CGA via intranasal route.*
- *In the dose optimization study, 10 mg/kg CGA i.n. showed significant reduction in infarct size, leakage of Evans blue, and brain water content.*
- *The treatment with CGA (10 mg/kg) significantly reduced the immunostaining of TNF-α, iNOS and Caspase-3 as compared to ischemia group.*
- *The treatment with ifenprodil and CGA significantly reduced the concentration of calcium in the brain samples and CSF compared to ischemia group. Also, CGA and 7-NI significantly reduced the level of nitrate in the cortex, cerebellum, hippocampi and CSF compared to ischemia group.*

Abstract

The ischemic cascade is initiated in the hypoperfused region of the brain that leads to neuronal cell death. Identification of multi-target inhibitor against prominent molecular mediators of ischemic cascade might be a suitable strategy to combat cerebral ischemic stroke. The present study is designed to evaluate the neuroprotective efficacy of chlorogenic acid (CGA) in the global cerebral ischemic rat model. The effective dose of CGA was evaluated on the basis of reduction in cerebral infarction area percentage, Evans blue extravasation, and restoration of brain water content. The expression of tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), and caspase-3 was evaluated by immunohistochemistry and morphological and cellular alterations in the cortex were observed by brain histology. The level of glutamate,

calcium, and nitrate in different regions of the brain, as well as cerebrospinal fluid (CSF), was evaluated. The level of calcium and nitrate was compared with ifenprodil- an antagonist of N-methyl-D-aspartate receptor (NMDAR) and 7-nitroindazole- an inhibitor of neuronal nitric oxide synthase (nNOS) respectively. Further, molecular docking was performed to compare the inhibition potential of CGA against NMDAR and nNOS with their inhibitors. Dose optimization results revealed that intranasal administration of CGA (10mg/kg b.w.) significantly reduced the cerebral infarction area, Evans blue extravasation and restored the brain water content compared to ischemia group. It also significantly reduced the calcium, nitrate and glutamate levels compared to ischemia group in the cortex, hippocampus cerebellum, and CSF. Immunohistochemical analysis revealed that CGA significantly reduced the expression of TNF-α, iNOS, and caspase-3 as compared to the ischemia group. In molecular docking study, CGA displayed similar binding interaction as that of Ifenprodil and 7-nitroindazole with NMDAR and nNOS respectively. The current findings suggest that the treatment with CGA confers neuroprotection in global ischemic insult by inhibiting and downregulating the different molecular markers of cerebral ischemia.

8.1 INTRODUCTION

Research from last few years has been carried out for the development of novel treatments to protect the brain from the damage following the ischemic insult [10–12]. These studies have shown great promise in animal model of ischemic stroke but unfortunately, translation failed so far. Recent studies provide strong evidence that inhibition of molecular targets involved in neurodegeneration is an effective therapeutic approach. The preclinical studies have demonstrated that overexpression or abnormal function of N-methyl-D-aspartate receptor

(NMDAR) [70,280], and neuronal nitric oxide synthase (nNOS)/ inducible nitric oxide synthase (iNOS) [74–76] play detrimental role in cerebral ischemia-reperfusion injury. The inhibition of these molecular mediators might be a useful therapeutic strategy for neuroprotection. Therefore, a compound that possesses inhibition potential for molecular mediators of neurodegeneration may be developed as a neuroprotective drug [13].

Bilateral common carotid artery occlusion (BCCAO) - reperfusion is an acceptable experimental model to study the physiological, biochemical and molecular changes in the brain and to study the effect of the drug on biological targets under limited blood supply [246,281]. This model may be useful for testing the efficacy of CGA in reducing the brain infarct, blood-brain barrier damage, brain water contents as well as nitrate, calcium and glutamate level.

Previous molecular docking study has demonstrated that CGA can inhibit NMDAR and nNOS similarly to their inhibitor ifenprodil and 7-NI respectively. Also the brain pharmacokinetic study showed that CGA can successfully reaches the brain in higher concentration when administered via. the intranasal route as compared to the intravenous route [247].

Therefore, the present study has been carried out to evaluate the neuroprotective effect of CGA in bilateral common carotid artery occlusion-reperfusion induced cerebral ischemia in rats after administration of CGA via the intranasal route. The present study also used Ifenprodil (an antagonist of NMDA receptor) and 7-nitroindazole (7-NI, a selective inhibitor of nNOS) as reference compounds to compare the level of calcium and nitrate in rat brain respectively after CGA treatment.

8.2 MATERIALS AND METHODS

8.2.1 Reagents and Antibodies

CGA, ifenprodil, 7-NI were purchased from Sigma-Aldrich, Inc.; HPLC grade acetonitrile, water was purchased from Merck. Primary antibodies for iNOS (SC-651) are purchased from Santa Cruz, Biotechnology (USA), TNF-α (ab1793), Caspase-3 (ab44976) and calcium detection kit (ab102505) were obtained from Abcam Life Science (New Delhi, India). Secondary fluorescent tagged antibodies for IHC Cy2-conjugated and cy3-conjugated are bought from Merck Millipore and Chemicon respectively.

8.2.2 Animals

Inbred male albino rats (240 ± 30 g) were acclimatized for three weeks under conditions of controlled temperature (25±2 °C), constant humidity and a 12-h light/dark cycle with free access to standard laboratory food and water. The surgical procedures were performed as per the protocol for animal use and approved by the Central Animal Ethical Committee at Institute of Medical Sciences, Banaras Hindu University, Varanasi (Registration No. 542/02/ab/CPCSEA).

8.2.3 Study design

The present study is designed to evaluate the possible neuroprotective potential of CGA in a global ischemic rat model. The CGA is administered through intranasal route after 2h of occlusion. Both the common carotid arteries were occluded for 2h and reperfusion was carried out for 6h. Thereafter animals were euthanized, brains were isolated and processed for the evaluation of various parameters as shown in Fig. 8.1.

8.2.4 Global cerebral ischemia-surgical procedure

The surgical procedure to induce global cerebral ischemia using bilateral common carotid artery occlusion is discussed in section 5.2.3 of chapter 5. In this study, the artery is occluded for two hours followed by reperfusion of six hours. The sham and positive control group's animals received all surgical procedures except the ligation of CCAs.

8.2.5 Regional cerebral blood flow measurement

The procedure to measure the regional cerebral flow (rCBF) is discussed in section 7.2.4 of chapter 7.The LDF monitoring showed that rats subjected to BCCAO, rCBF was reduced by at least 80% of normal values after ligation of both CCAs. The rats those rCBF did not exhibit a reduction of 80% were excluded from the study.

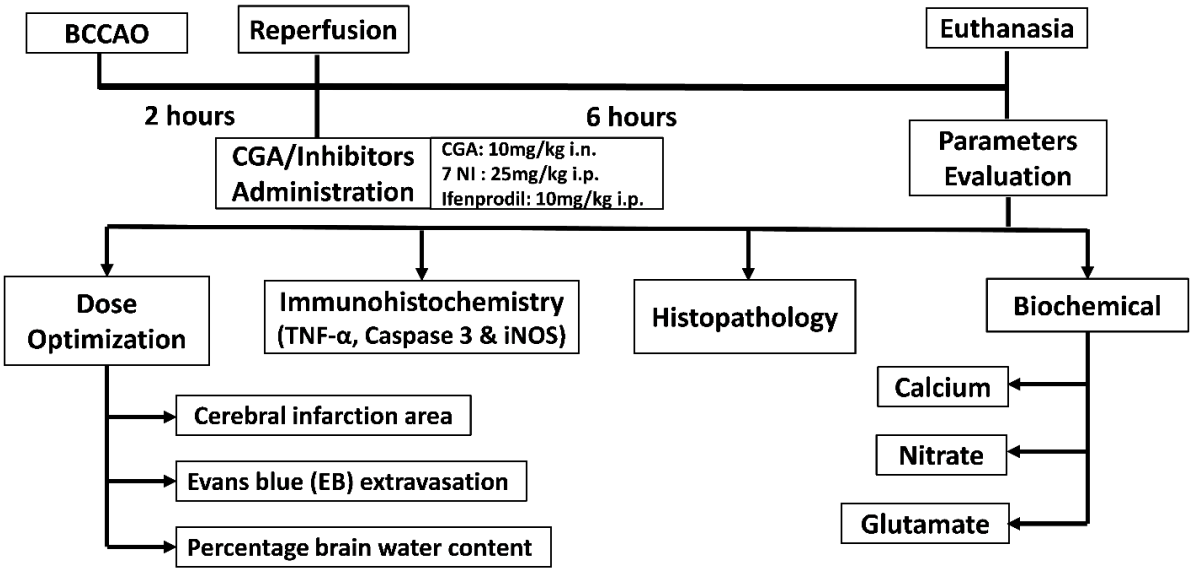

Fig. 8.1: Workflow diagram of experimental design

8.2.6 CGA/inhibitors administration

CGA was dispersed in 0.1M phosphate buffer (PB; pH 7.4) and administered through intranasal route. Briefly, CGA of strength 125 mg/ml, dose 10 mg/kg and dose volume 80 µl/kg was prepared, protected from light and immediately used for the intranasal dosing. Single dose of 10 mg/kg with dose volume 80 µl/kg was achieved by administration of 40 µl/kg of CGA dispersion in both nostril of rat via a hollow tubing (inserted upto10 mm) attached to a 25 ml Hamilton syringe [247]. 7-NI (25 mg/kg), dissolved in peanut oil and was administered intraperitoneally [213,282]. The Ifenprodil (10 mg/kg) was dissolved in normal saline and was administered intraperitoneally [283]. The treated group was received a respective dose of CGA/inhibitors after 2h of occlusion.

8.2.7 Experiment A: Dose optimization

- **Grouping**

A dose optimization study was performed to determine the effective neuroprotective dose of CGA. Three parameters, i.e., cerebral infarction area, Evan's blue extravasation and % brain water content were chosen for this study. Three doses of CGA (5mg/kg, 10mg/kg and 15mg/kg b/w.) were evaluated to determine the effective dose. A total of 50, 30 and 25 rats were utilized for the determination of cerebral infarction area, Evan's blue extravasation and % brain water content respectively. The rats were equally and randomly divided into five groups consisting of a sham, ischemia, ischemia + CGA (5mg/kg b.w.), ischemia + CGA (10mg/kg b.w.), and ischemia + CGA (15mg/kg b.w.). The ischemia group received vehicle (0.1M phosphate buffer, dose volume 80 µl/kg) after 2h of occlusion via intranasal route.

- **Estimation of cerebral infarction area**

The effect of CGA on cerebral infarction area was evaluated using triphenyltetrazolium chloride (TTC) staining [276]. The procedure of cerebral infarction area estimation using TTC staining is discussed in section 7.2.6 of chapter 7.

- **Evans blue (EB) extravasation**

The effect of different dosage of CGA on the integrity of the blood-brain barrier (BBB) was investigated using Evans blue extravasation (EBE) [278] with slight modification. The EBE procedure is discussed in section 7.2.7 of chapter 7.

- **Brain water content**

The % brain water content of all groups was determined by the wet- weight and dry-weight method using the formula 100*(wet weight - dry weight)/wet weight [284]. The rats were decapitated under anesthesia and brain was removed from the skull. The wet weight was determined immediately and dry weight was determined after drying the brain in a desiccating oven at 100°C for two days to obtain a constant weight.

8.2.8 Experiment B: Immunohistochemistry/ Histological investigation

- **Grouping**

For immunohistochemical/Hematoxylin and Eosin staining, 32 rats were randomly divided into four groups for each parameter i.e. sham (n=4), sham + CGA (10mg/kg) (n=4) as positive control group, ischemia (n=4) and ischemia + CGA (10mg/kg) (n=4). The ischemia group received vehicle (0.1M phosphate buffer, dose volume 80 µl/kg) after 2h of occlusion via intranasal route.

- **Immunohistochemistry**

Immunohistochemical staining of TNF-α, iNOS, and Caspase-3 was performed in cerebral cortex using standard procedure [285]. Briefly, rats from each group were first anesthetized and perfused intracardially with 0.9% saline (chilled) and 4% paraformaldehyde (chilled) prepared in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were taken out after decapitation and kept in 10% paraformaldehyde overnight and further transferred into sucrose solution 10, 20, 30% sucrose solution. Then, 20 μm thick 6-8 brain sections were cut coronally using a cryomicrotome (Leica, Wetzlar, Germany). Sections were then washed with 0.01% M PBS (pH 7.4) at 10 min interval and then blocked with 10% normal goat serum in PBS 0.3% Triton-X 100 and 1% BSA in PBST, i.e., blocking reagent for about 1 h. The sections were further incubated with a primary antibody (TNF-α, iNOS, and Caspase-3) for 16 hr at 4°C. Further, washing was done with PBS and 1% BSA-PBS respectively two times each to remove unbound primary antibodies and then incubated with Cy2-conjugated secondary antibody (for anti-mice primary) and Cy3-conjugated secondary antibody (for anti-rabbit primary) prepared in 1% BSA-PBS for 1h at RT. Then sections were washed thrice with 1% BSA-PBS at 3 min interval. Finally, sections were washed three times with PBS and then mounted on slides using polyvinyl alcohol mounting medium with DABCO anti-fading (Fluka analytical). The images were taken under a fluorescent microscope (Nikon Instruments Inc.). Immunofluorescence was analyzed by Image J software (NIH United States). The results were reported as mean integrated fluorescent value (IFV).

- **Histological investigation (Hematoxylin and Eosin Staining)**

Hematoxylin and Eosin Staining of rat brain was carried out according to Cardiff et al., 2104 [279]. Briefly, the brain was carefully removed after transcardial perfusion with chilled saline

and immediately fixed with 10% formalin solution for the histology study. Tissue specimens were embedded in paraffin and sectioned at 5 μm for staining with hematoxylin and eosin and was analyzed with a fluorescent microscope (Nikon Instruments Inc.).

8.2.9 Experiment C: Biochemical parameters (estimation of nitrate, calcium and glutamate level)

- **Grouping**

For the determination of nitrate, calcium and glutamate levels in cortex, hippocampi, cerebellum and CSF samples of rat's brain, 60 rats were randomly divided into seven groups i.e. sham (n=12), ischemia + CGA vehicle (n=12; received 0.1M phosphate buffer, after 2h of occlusion via intranasal route), ischemia + 7-NI vehicle (n=6; received peanut oil, 5ml/kg i.p.), ischemia + ifenprodil vehicle (n=6; received normal saline 5ml/kg i.p.), ischemia + 7-NI (n=6; received 7-NI, 25 mg/kg, i.p.), ischemia + Ifenprodil (n=6; received Ifenprodil, 10 mg/kg, i.p.) and ischemia + CGA (n=12 received CGA, 10mg/kg i.n.).

- **CSF sampling**

The CSF (≈50 μl) of anesthetized rats was collected with the help of 23 G needle in labeled tubes from the cisterna magna without making any incision in this region according to procedure demonstrated by Nirogi et al., 2009 (Nirogi et al. 2009).

- **Estimation of Nitrate level**

The level of nitrate in cortex, hippocampi, cerebellum, and CSF were determined using HPLC-UV method as discussed in chapter 5.

- **Determination of calcium level**

The colorimetric calcium detection kit (ab102505; Abcam) was used for the determination of calcium concentration in the rat brain. The standards and sample were prepared according to the assay protocol. Briefly, 10 mg of brain tissue samples (cortex, hippocampi, cerebellum) were washed in cold PBS solution and re-suspend in 500µl of Calcium Assay Buffer. Tissues were homogenized using tissue grinder on ice. Samples were centrifuged for 5 minutes at 4°C at 2100 RCF and supernatants were collected and transferred to a clean tube. The mixture of 50µL sample, 90µL of the Chromogenic Reagent and 60µL of Calcium Assay Buffer was incubated for 10 minutes protected from light. For CSF, 50µL of the volume was adjusted by diluting the 20µL CSF in MilliQ water. The absorbance was measured on a microplate reader (OD575 nm) and concentration was determined using a standard curve.

- **Glutamate Concentration**

The glutamate neurotransmitter concentration in cortex, hippocampi, cerebellum, and CSF was performed using HPLC-UV method reported by Wu et al., 2014 with slight modification [286]. Briefly, the hippocampi (0.2 gm), cerebellum (0.5 gm), and cortex (0.5 gm) were extracted and homogenized in 0.5, 1 and 1 ml of chilled saline solution (0.9%). The homogenates were centrifuged for 10 min at 4°C at 12,000 RCF and the supernatant was transferred in clean tubes after filtration by 0.22µm filter and kept in -20°C for further processing (derivatization). The well-mixed solution (10µl sample, 20µl of acetonitrile, 2.5µl of NBD-F working solution and 17.5µl of borate buffer solution) was allowed to react for 7 min at 60°C in the water bath, protected from light. After incubation, samples were cooled at room temperature and 10µl of the solution was injected into the HPLC system. The mobile phase was composed of 0.02 M phosphate buffer-acetonitrile (84:16, v/v) and the flow rate was set at 1.0 ml/min. The level of

glutamate was monitored at a wavelength of 472 nm throughout the experiments and concentration was determined using a standard curve.

8.2.10 Statistical Analysis

The biochemical parameters data were analyzed by two-way ANOVA using Bonferroni's post-hoc test, immunofluorescence data and dose optimization parameters were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman– Keuls test using Graph Pad Prism 7.0 software. The results are expressed as the means ± SD. P-values <0.05 were considered statistically significant.

8.3 RESULTS

8.3.1 Experiment A: Dose Optimization

CGA attenuates 2/6 h I/R injury-induced cerebral infarction area, BBB disruption, and brain swelling

The effective neuroprotective dose of CGA was determined on the basis of reduction in infarct area, Evans blue extravasation and brain water content of rats subjected to 2 hours of occlusion followed by 6 hours of reperfusion (2/6 h I/R) injury.

The 2/6 h I/R injury produced marked infarcts in both cortical and subcortical regions of rat brain. The treatment with CGA at 5, 10, and 15 mg/Kg i.n. doses produced a marked reduction in infarct area by 17 ($p<0.01$), 65 ($p<0.001$) and 56 ($p<0.001$) percentage respectively, relative to the ischemia group (Fig.8.2a). There was no significant difference observed in reduction of cerebral infarction area percentage between 10 mg/kg and 15 mg/kg dose.

The Evans blue dye assay was performed with different doses of CGA in 2/6 h I/R injury (Fig. 8.2b). Similar to previous findings on cerebral infarction, the treatment with CGA at 5, 10, and

15 mg/Kg i.n. doses significantly reduced the leakage in the injured brain compared to ischemia group and the decrease in the Evans blue extravasation was approximately 41 ($p<0.05$), 79 ($p<0.01$) and 76 ($p<0.01$) percentage respectively. There was no clear difference between the 10 and 15 mg/kg CGA treated groups ($p<0.05$).

Brain swelling is a major outcome due to an increase in brain water content. Treatment with 10 and 15mg/kg CGA significantly reduced the brain water content as compared to ischemia ($p<0.01$) (Fig. 8.2c). There was no significant difference between the 5 mg/kg CGA treatment and ischemia groups; sham and 10 mg/kg CGA treated groups as well as between the 10 and 15 mg/kg CGA treated groups ($p<0.05$). It is evident that 10 and 15mg/kg CGA significantly restored the brain water content near to normal value.

In the dose optimization study, 10 mg/kg CGA i.n. showed a significant reduction in infarct size, leakage of Evans blue, and brain water content. Therefore, 10 mg/kg CGA was selected as an effective neuroprotective dose for further studies.

8.3.2 Experiment B: Immunohistochemistry/ Histological investigation

CGA reduced the expression of iNOS, TNF-α, and caspase -3 in the cortex

We have studied the effect of CGA (10 mg/kg, i.n.) treatment in 2/6 h I/R injury on the expression of nitrosative stress marker (iNOS), apoptotic marker (caspase -3), and neuroinflammatory marker (TNF-α) within the cortex (Fig. 8.3). Immunohistochemical staining revealed that the expression of TNF-α ($p<0.001$), iNOS ($p<0.001$) and Caspase-3 ($p< 0.001$) was significantly enhanced in the ischemia group as compared to sham and positive control groups. The treatment with CGA (10 mg/kg) significantly reduced the immunostaining of TNF-α ($p < 0.001$), iNOS ($p < 0.001$) and Caspase-3 ($p < 0.001$) as compared to ischemia

group. There was no significant difference observed in the expression level of TNF-α, iNOS and caspase-3 between sham and positive control (sham + CGA 10mg/kg) groups.

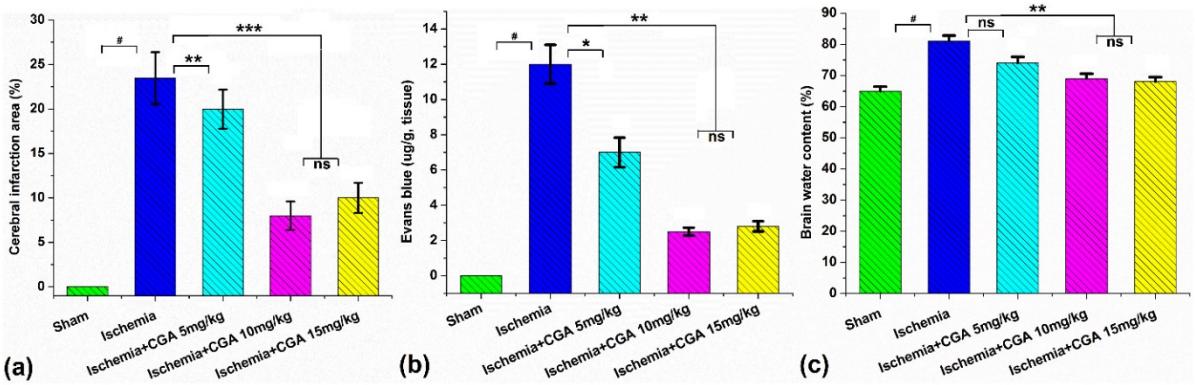

Fig. 8.2: Dose optimization parameters. (a) Cerebral infarction area percentage, (b) Evans blue extravasation and (c) Brain water content percentage at different doses of CGA (5, 10 and 15 mg/kg b.w.). Data are expressed in terms of mean ± SD. #p < 0.001; ∗p < 0.05, ∗∗p < 0.01, and ∗∗∗p < 0.001, ns: non-significant.

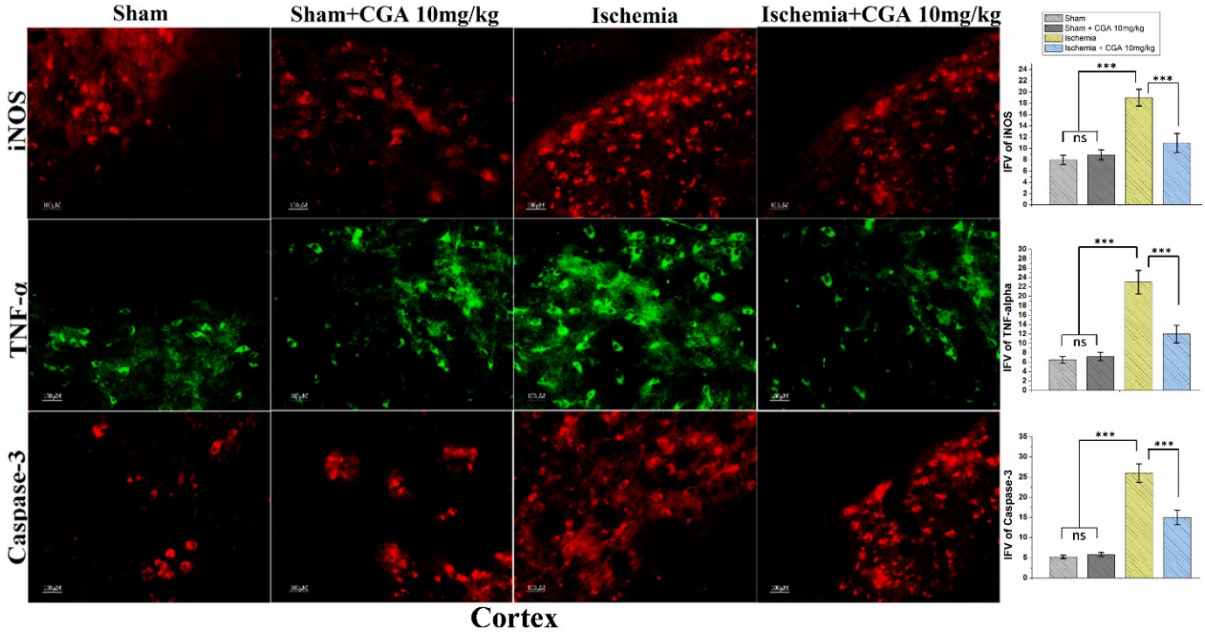

Fig. 8.3: Immunohistochemistry of iNOS, TNF-α, and caspase-3 in the cortex of rat with 10X magnifications after staining. The expression of iNOS, TNF-α, and caspase-3 were significantly increased in the cortex of ischemia group compared with the control group, whereas the expression of iNOS, TNF-α, and caspase-3 was significantly reduced in CGA-treated group compared to ischemia group. Data are expressed in terms of mean ± SD (∗∗p < 0.01, and ∗∗∗p < 0.001).

CGA ameliorates the 2/6 h I/R injury-induced morphological and cellular alterations in the cortex

Hematoxylin and eosin (H&E) stains were used to evaluate the morphological and cellular alteration caused by ischemic condition and effect of CGA treatment. The H&E staining demonstrated that the infarction area characterized by the presence of vacuolation, small nuclei and deformed with cracked cytoblasts in the ischemia group. Treatment with 10mg/kg CGA ameliorates the alteration induced by ischemia (Fig. 8.4).

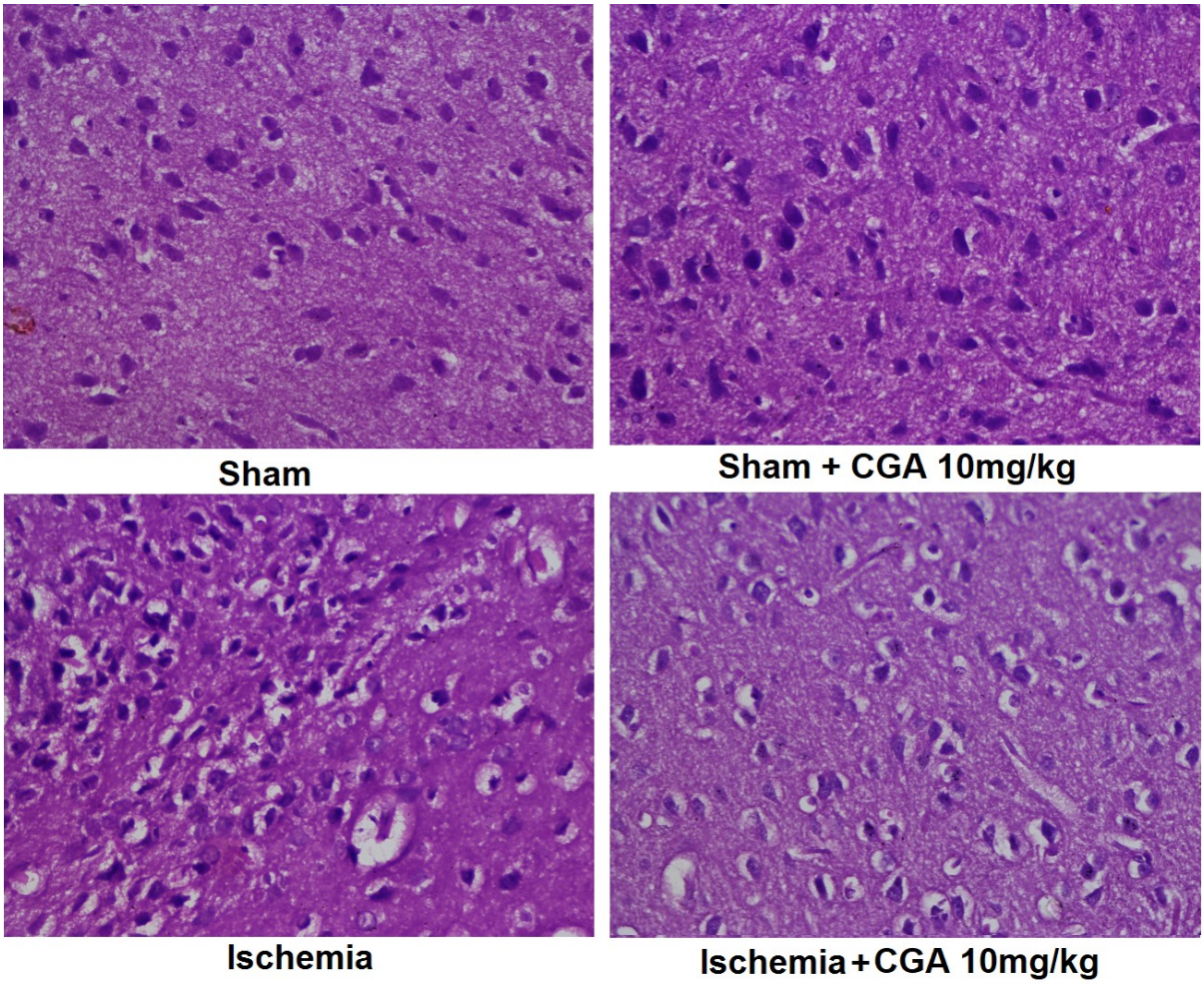

Fig. 8.4: Hematoxylin and eosin (H&E) staining of cortex.

8.3.3 Experiment C: Biochemical parameters

CGA reduced the concentration of calcium, nitrate, and glutamate in I/R rat brain

The level of calcium in the cortex, cerebellum, hippocampi, and CSF was significantly higher in the ischemia group compared to sham ($p<0.001$). Treatment with ifenprodil and CGA significantly reduced the concentration of calcium in the brain samples and CSF compared to the ischemia group ($p<0.001$) (Fig 8.5a). There was no significant difference between ifenprodil and CGA treated groups ($p<0.05$).

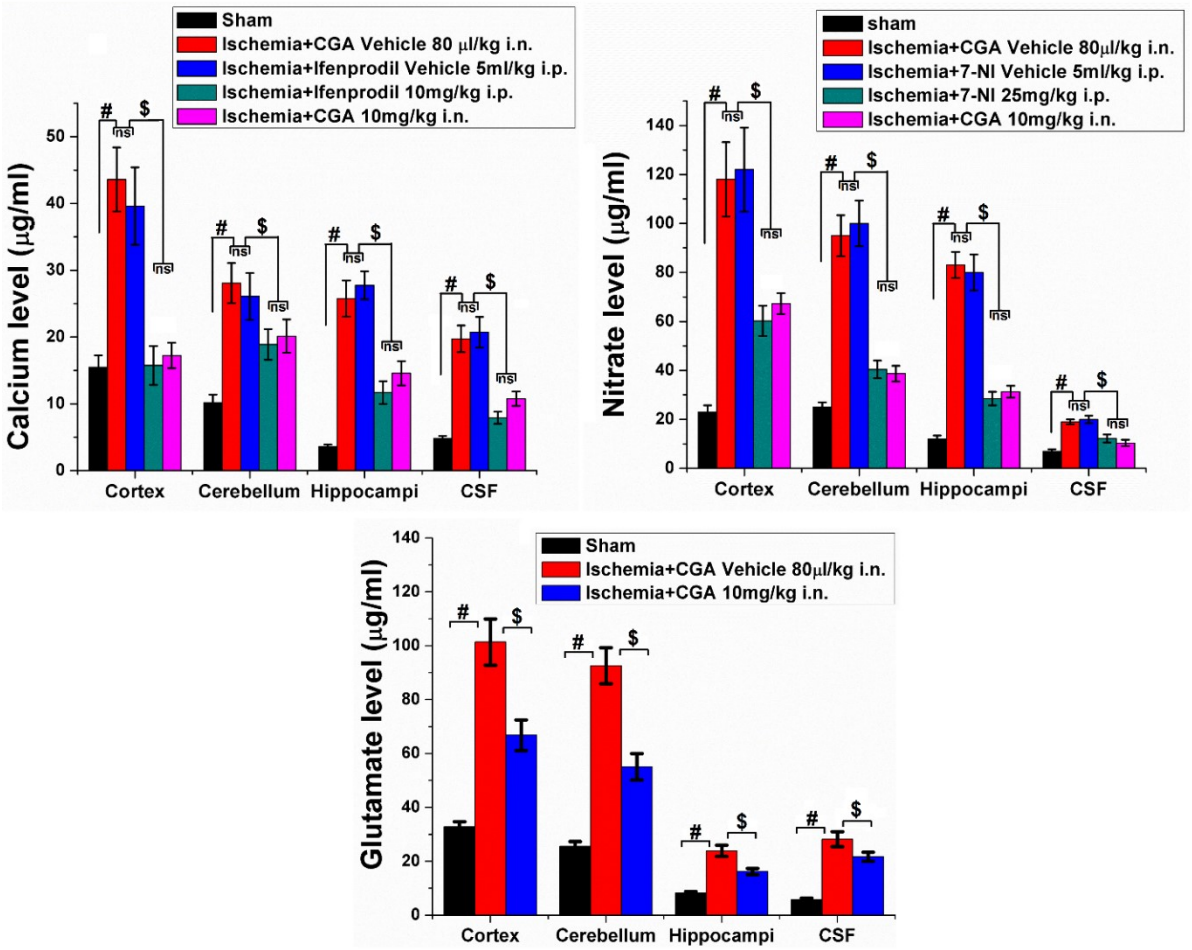

Fig. 8.5: Biochemical parameters. (a) Calcium level, (b) Nitrate level and (c) Glutamate level in cortex, cerebellum, hippocampi, and CSF. Data are expressed in terms of mean ± SD; ns: non-significant; # ischemic group compared with sham, $p < 0.05$ and $ Ischemia+vehicle group(s) compared with treated group(s), $p < 0.05$.

The over-activation of nNOS and iNOS during I/R injury was indirectly evaluated by determination of nitrate concentration in different brain samples and CSF. A marked increase in the concentration of nitrate was observed in the ischemia group compared to sham ($p<0.001$). Treatment with CGA and 7-NI significantly reduced the level of nitrate in the cortex ($p<0.001$), cerebellum ($p<0.001$), hippocampi ($p<0.001$) and CSF ($p<0.01$) compared to ischemia group. There was no significant difference observed in the nitrate concentration between 7-NI and CGA treated groups (Fig 8.5b).

The level of glutamate in the cortex, cerebellum, hippocampi, and CSF was increased 4, 4, 3.5 and 5.2 times respectively in the ischemia group compared to sham ($p<0.001$). Administration of 10mg/kg CGA 2h after the onset of ischemia significantly lower the glutamate concentration in the cortex ($p<0.001$), cerebellum ($p<0.001$), hippocampi ($p<0.01$) and CSF ($p<0.01$) compared to the ischemia group (Fig 8.5c).

8.4 DISCUSSION

The present study demonstrated that CGA has neuroprotective effects on cerebral infarction, brain edema, and BBB damage in 2/6 h I/R induced injury in rat brain. It suppressed the expression of TNF-α, iNOS, and caspase-3 as well as reduced the level of glutamate, calcium, and nitrate in different brain regions and CSF.

Intranasal (IN) administration directly targets the drug into the brain through the olfactory epithelium, bypassing BBB and first-pass metabolism [224]. Therefore, this route of drug administration is safe and can be used to deliver neurotherapeutics to the CNS for the treatment of neurological disorders with less systemic exposure with a minimal side effect. Previously,

brain penetration study revealed that the concentration of CGA in the brain is 4 times higher at 30 min in IN administration compared to (intravenous) IV administration [247]. In the present study, we have used IN administration of CGA to evaluate its neuroprotective potential at three doses and found the effective neuroprotective dose was 10 mg/kg.

Although, permanent ligation of the bilateral CCA has shown to induce severe ischemic damage [287], a temporary occlusion of the bilateral CCA followed by reperfusion also induce ischemic lesions, brain edema and BBB opening in the rat brain [92,288,289]. The incidence and severity of the ischemic lesion with BBB disruption depend on the duration of occlusion. Also, intermittent repetitive ischemia is more hazardous and caused lesions in the hippocampus, caudoputamen, neocortex, and thalamus [289]. In this study, 2h of occlusion followed by 6h of reperfusion causes a large infarction in rat brain in the ischemia group (Fig 8.2a) and treatment with CGA effectively reduced the cerebral infarction area compare to the ischemia group.

The BCCAO caused reduction of CBF up to 94% that disturb the ion-homeostasis resulting in the BBB dysfunction [288]. The proteolytic gelatinase enzymes matrix metalloproteinases (MMP)-2 and MMP-9, are central mediators of BBB disruption in ischemic insult [290]. Brain edema in ischemic insult is closely associated with BBB disruption [291] and characterized by the accumulation of fluid in the extra and intracellular spaces with an increase in the brain volume [288]. Previously, CGA has been reported to protect the integrity of BBB by inhibiting the expression of MMP-2 and MMP-9 mRNA and protein [218]. In this study, we also found that treatment with CGA decreases the Evans blue extravasation compared to ischemia group. The brain water content was measured as characteristics of cerebral edema and it was found that both 10 and 15 mg/kg CGA significantly restored the brain water content. The evaluation

of the above parameters clearly shows that both the doses 10 and 15 mg/kg effectively combated the 2/6 I/R-induced damage in rat brain. It should be mentioned here that 10mg/kg CGA is preferred over 15mg/kg of dose because this dose has three advantages: effective for neuroprotection, ease of solubility in PB and intranasal administration. Therefore, this dose is used for further experiments.

Inflammation plays important role in the progression of ischemic stroke, although the underlying mechanisms are yet to be explored [292]. The most studied pro-inflammatory mediators related to inflammation in acute ischemic stroke are TNF-α, a pro-inflammatory cytokine [292,293]. The pro-inflammatory and cytotoxic factors usually help cells by rescuing them from invading pathogen. However, under continued production, they might lead to neurotoxic effects resulting in the damage of cell [293]. The expression of inducible NOS (iNOS) is calcium independent and mediated by various cytokines including TNF-α, exotoxins and bacterial endotoxins. Its overexpression in ischemic insult, closely associated with the apoptosis by producing a large amount of nitric oxide (NO) [294]. The ischemia also induced overexpression of caspase-3, an apoptotic enzyme that regulates neuronal apoptosis in both ischemic core and penumbra [295,296]. Previous studies have revealed that inhibition of these markers has successfully reduced the ischemic damage [166,297,298]. IHC study using an antibody against these ischemic markers in cerebral cortex revealed that the enhanced levels of TNF-α, iNOS, and caspase-3 in ischemia group was significantly reduced by treatment with 10mg/kg of CGA. The present study explores the neuroprotective role of CGA that can also be attributed to its ability to reduce the expression of TNF-α, iNOS, and caspase-3. Ischemic damage also results in morphological and cellular changes in brain tissue [299]. Study of histological changes of brain tissue was performed by HE staining and the results showed a

marked reduction of vacuolation and lower number of shrunk nuclei along with cracked cytoblasts in the 10mg/kg treated group as compared to the ischemia group.

In cerebral ischemia, a reduction of cerebral blood flow, oxygen, and nutrients lead to loss of ionic homeostasis which results in the release of excitatory neurotransmitter especially glutamate [300]. Increase in glutamate concentration causes the overactivation of NMDAR, a type of ionotropic glutamate receptor, thus causing neuronal excitotoxicity [301]. As a result, there is a rapid influx of calcium ions into the neuronal cell which simultaneously initiates the ischemic cell death pathway (Fig. 2.1). Ifenprodil, a well-established allosteric inhibitor of NMDAR[153] is used in this study to compare the effectivity of CGA on calcium concentration in different brain regions and CSF. It was observed that both ifenprodil (10mg/kg, i.p.) and CGA (10mg/kg, i.n.) significantly reduced the elevated calcium level observed in ischemia group in the cortex, hippocampi, cerebellum, and CSF. No significant difference was observed between CGA and ifenprodil treated groups which establishes the ability of CGA to lower the calcium concentration with potency as same as ifenprodil. In addition, it was also observed that CGA significantly lowered the glutamate concentration in cortex, hippocampi, cerebellum, and CSF as compared to the ischemia group. These findings were in accordance with some previous studies that demonstrated that CGA confer protection against L-glutamate induced cortical neurons injury by reducing the level of intracellular calcium concentration [302][303].

Over accumulation of calcium in neuronal cells leads to further activation of nNOS resulting in an increased concentration of nitric oxide in brain tissue[296]. Increased NOS activity leads to the disruption of blood brain barrier and edema formation in acute ischemic stroke [304]. Overproduction of NO after brain ischemia resulting increased cerebral vascular injury and

infarct size in MCAO rat model [305]. It is evident that NO react with superoxide and formed peroxynitrite (a potent oxidant) which is associated with neuronal cell death [161]. The activation of iNOS is also responsible for NO overproduction which is associated with neuronal cell apoptosis and iNOS inhibition reduce the severity of ischemia-reperfusion injury in rats [306]. Since the nitric oxide is a very short-lived molecule and is converted into nitrate and nitrite within few seconds, estimation of nitrate and nitrate is generally carried out to indirectly measure the total NO concentration. However, nitrite also has a very short half-life which imposes difficulty in its reliable measurement. Therefore, in this study, the concentration of nitrate was measured using 7-NI, a selective inhibitor of nNOS [213]as a reference drug. Both CGA (10mg/kg, i.n.) and 7-NI (25mg/kg, i.p.) cause significant reduction of nitrate level in various parts of rat brain (cortex, hippocampi, cerebellum) and CSF. The lack of any significant difference between CGA and 7-NI groups suggest the ability of CGA to reduce the nitrate concentration during ischemic damage is similar to that of 7-NI.

Our findings suggest that the CGA efficiently restored calcium, nitrate level, and its efficiency was comparable to the known NMDAR and nNOS inhibitors. We further explore the ability of CGA to inhibit the NMDAR and nNOS using molecular docking simulation. Molecular docking is the most frequently used structure-based drug designing strategies and an essential tool in drug discovery [103]. Molecular docking Genetic algorithm is an application of the stochastic search that performs quantitative predictions of binding energetics and provides rankings of docked ligands based on their binding affinity for receptor's pockets [307]. It also provides deep insight into hydrogen bond, hydrophobic interaction, pi-pi interactions between ligand-receptor complexes [308]. The combination of experimental and computational strategies has been of pronounced assessment in the identification of novel drug compounds.

In the present study, we observed that CGA binds to the NMDAR in a similar fashion as compared to ifenprodil. Though the binding energy of CGA was higher than ifenprodil, it bound to the NMDAR ATD by forming the H-bonds with Leu135, Gln110, Ser132, Tyr109 and Glu236 which is similar to ifenprodil. CGA also binds to nNOS heme domain with binding energy lower than 7-NI establishing that it has a higher affinity toward nNOS as compared to 7-NI. Similar to 7-NI, CGA formed H-bonds with Trp587, Met589, and Gly586. CGA also formed a greater number of H-bonds with nNOS than 7-NI which establishes its higher affinity toward nNOS. The molecular simulation study demonstrates that CGA has the ability to simultaneously inhibit both NMDAR and nNOS which might be responsible for its neuroprotective efficiency.

In the present study, CGA significantly reduced the level of calcium with potency as same as ifenprodil and level of nitrate with effectiveness same as 7-NI in the cortex, cerebellum, hippocampi, and CSF in the ischemic rat model. This *in vivo* study is positively supported by the findings of molecular docking study. Therefore, we can say that CGA has a dual inhibition potential for NMDAR and nNOS. However, neither intracellular calcium accumulation nor nitrate overproduction is solely governed by NMDAR and nNOS respectively. During ischemic condition, translocation of extracellular calcium into the intracellular space is facilitated by various ion channels including NMDAR, unspecified reactive oxygen species-activated ion channels, voltage operated and agonist operated ion channels [309]. Additionally, both nNOS and iNOS are responsible for the overproduction of nitric oxide [74]. Therefore, further study is needed to explore the inhibition potential of CGA for other molecular mediators of neurological dysfunction as well as its exact mechanism of antiapoptotic and anti-inflammatory properties.

8.5 CONCLUSION

Our findings suggest that CGA can be used as a potent neuroprotective agent in global ischemic condition. After treatment, it confers neuroprotective effects mainly by downregulating the expressions of proinflammatory and apoptotic markers in the cortex as well as by reducing the cerebral lesion, BBB disruption, and brain edema. The neuroprotective ability of CGA might be attributed to its dual inhibition of NMDAR and nNOS that is supported by *in vivo* and molecular docking studies. From the above findings, it can be concluded that the ability of CGA to inhibit and downregulate different molecular markers of cerebral ischemia can be exploited for designing a multi-target inhibitor in neurotherapeutic for combating cerebral ischemic stroke.