Materials and Methods

3.1 Materials:

3.1.1 Drugs:

Progesterone and estrogen were received as gift samples from Sun pharma Ltd. Vadodara. Co-enzyme Q-10 was obtained from Troikka pharmaceuticals Ltd. Ahemdabad. α-Lipoic acid and Trimetazidine hydrochloride was received as a gift sample from Alembic Pharma Ltd., Vadodara and Unichem Pharmaceuticals Ltd., Mumbai respectively. The raw material (leaves) of Banaba (*Lagerstroemia speciosa L*) was provided by Cherain Chemicals Ltd., Vadodara for this study.

3.1.2 Chemicals:

Superoxide dismuatse (SOD), crystalline beef liver catalase (CAT), 1,1,3,3-tetrahydroxy-propane, glutathione (GSH), Epinephrine Hydrochloride, Triphenyl tetrazolium chloride(TTC), hexadecyl trimethyl ammonium bromide (HTAB), ortho-dianisidine dihydrochloride, poly L- lysine, hydrogen peroxide were purchased from Sigma Aldrich; U.S.A.

A 1 Kb DNA marker was obtained from Fermentas, Germany. RNase A, ethidium bromide, agarose were procured from Himedia Laboratories Pvt. Ltd, Mumbai. Thiobarbituric acid, tris buffer, sucrose, ATP, reduced glutathione, 5,5'-dithiobis (2-nitro benzoic acid), bovine serum albumin, Evans blue, trichloroacetic acid were purchased from Himedia. Folin's phenol reagent was procured from S.D. Fine Chemicals, Mumbai, India.

The diagnostics kit used for the Creatine kinase estimation was purchased from Rickon diagnostics Pvt.Ltd., Vadodara. All other chemicals used in the study were of analytical grade.

3.1.3 Plant material and preparation of extract:

Leaves of Banaba (*Lagerstroemia speciosa L*) were collected and shed dried for preparation of the extract. Botanical Survey of India, Pune, authenticated the specimen. The specimen number is BSI/WC/Tech/2007/811. The extract was prepared by cold maceration. 1 kg raw material was macerated with 2.5 lit. ethanol for 72 h. Extract was concentrated and dried under vacuum (1.3% w/w). Further extract was standardized with

respect to Corosolic acid using HPTLC (Camage, USA). Corosolic acid content was found to be 1%.

3.1.4 Animals:

The experimental procedures were performed using albino rats of Sprague Dawley strain of either sex weighing 280-320 g were procured from Central Animal House facility of M.S. University of Baroda. All the study protocols were approved by The Institutional Animal Ethics Committee, M.S. University of Baroda that follows the NIH Guidelines for care and use of animals (NIH publication No. 85-23, revised in 1996). All the animals were housed in a room at temperature of 24 ± 1 °C and 12 h light and dark cycle. Animals were fed with standard pallet diet and water *ad libitum*.

3.2 Experimental procedure:

Two types of ischemia/reperfusion (I/R) injury were carried out i.e. *1.* Myocardial I/R using left coronary artery ligation (LCA) model and *2.* Cerebral I/R by Middle Cerebral artery occlusion (MCAO) model to study the effect of various hormones and their combinations with antioxidants.

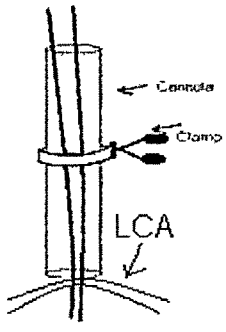

Fig. 4 Showing tightening of canula

3.2.1 Myocardial ischemia/reperfusion injury induction by left coronary artery (LCA) ligation: The myocardial I/R injury was produced by LCA ligation model[354]. The animals were anaesthetized with Ketamine (100 mg/kg i.p.) + Xylazine (7 mg/kg i.m.) and then were artificially ventilated (Ugo Basile Rodent Ventilator, Model 7025). The body temperature was maintained at 37 ± 1 °C during the experiment. The heart was exposed by removing the oblong portion of chest wall and sternum after performing the thoracotomy in 4th intercostals area. A tie of 6.0 silk sutures was placed around the LCA after the identification of location. The tie was then passed through a plastic cannula to allow occlusion (60 min) and reopening (60 min) of the vessel to induce ischemia and reperfusion respectively. The Sham operated animal underwent the same procedure except tightening and releasing the cannula. The carotid artery was cannulated for the measurement of hemodynamic parameters using transducer (SL 13L, Biopac Systems)[355]. The hemodynamic parameters observed were systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), dp/dt. The electro-cardiographic

parameters like R-amplitude, ST elevation and ST-segment duration were observed. All the data were analyzed using Biopac Student Lab Pro software (Version 3.6.7).

This protocol of 60 minute ischemia was based on the earlier reports[356], whereas the protocol with 60 min reperfusion was preferred to study antioxidant effects of drugs as the levels of thiobarbituric acid reactive substances i.e. malondialdehyde as a marker of lipid peroxidation (LPO) peaks at 60 minutes of reperfusion. The experiment was performed in three sets of animals (n=6) for every group. The first set was used for the measurement of infarct area. Second set was utilized for estimation of oxidative stress parameters (SOD, GSH, CAT and LPO) and measurement of myeloperoxidase (MPO) activity. Third set was employed for DNA fragmentation analysis and histological observations.

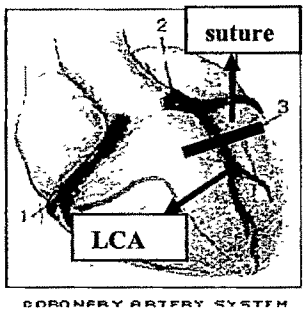

Fig. 5 Showing silk suture passed underneath LCA for induction of ischemia

To study the effect of estrogen, ovariectomy was performed on the animals at the age of around 10-12 week. The animals were anaesthetized, the ovary was accessed through a lateral abdominal incision, and the ovarian artery and vein were clamped with a surgical clip and ligated. The ovary was then resected, surgical wounds were sutured and the animal allowed to recover for 2 to 4 weeks before performing the I/R protocols[357].

Protocol -1

Determination of myocardial infarct size and area at risk:

The animals were sacrificed after 2 h of I/R injury for removing the hearts. The LCA was briefly re-occluded and Evans Blue (2%, 3 ml) was forced retrogradely through aorta to allow perfused and non-perfused areas of the heart to be distinguished. Thereafter, the heart was frozen and sectioned (1-1.5 mm) perpendicular to apex-base axis to distinguish between the area at risk and infracted myocardium. The sections were incubated in TTC (0.5 mg/ml for 20 min at 37 °C). The red stained area was considered as area at risk (AAR). The blue stained portion was non ischemic myocardium and unstained whitish portion was infarct size.Total area, area at risk (AAR) and ischemic area (IS) of each image was measured five times and the mean of each area of every slide was calculated and determined by computer based Image J software 1.30 V (rsb.info.nih/ij.)[354]. The hemodynamic parameters like SBP, DBP, MAP and the maximal pressure change rate ($\pm dp/dt$) were observed at three intervals (Baseline, End-Ischemia, and End-Repefusion).

ECG parameters studied during the study were ST elevation and ST prolongation induced by I/R in various groups of animals. (Section 3.3)

Protocol -2

The biochemical and antioxidant parameters for estimations of various markers in heart were carried out as described in section **3.4.2**. MPO activity was measured according to the procedure given in section **3.4.5**.

Protocol -3

DNA fragmentation analysis and histological observations were performed as described in section **3.4** and **3.6** respectively.

Exclusion criteria

The animals which were showing sustained fall in blood pressure and succumbed to the experimental procedure were excluded from the study. The animals were replaced and the total number of the animal in each group was evenly maintained.

3.2.1.1 Groups and acute treatment schedule for drug administration

Progesterone and estrogen were administered to the animals in acute study treatment schedule. In the present protocol, these hormones were studied individually and in combination with various antioxidants to observe its synergistic activity if any on the reperfusion injury.

Estrogen was studied on ovariectomised animals through out the study. The Sham operated control (SO) animals underwent the LCA procedure except tightening and releasing the cannula thus emulating the surgical stress. The vehicle treated reperfusion injury (I/R control) group was studied to observe the effect of vehicle in the study. The drugs were administered individually or in combination after reperfusion injury in this study.

3.2.1.1.1 Progesterone (PG) alone treatment:

Progesterone was dissolved in pea nut oil (vehicle) and administered by i.p. route 30 min after induction of ischemia. Vehicle treated group was considered as Reperfusion injury control in all the studies. Vehicle was administered (2 ml/kg i.p.) 30 min after induction of ischemia. Following groups were involved in the study.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg i.p.)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Progesterone (PG) treatment (2 mg/kg i.p.) after 30 min of ischemia induction.

3.2.1.1.2 Lipoic acid (Lipo) alone treatment:

Lipoic acid was dissolved in pea nut oil (vehicle) and administered by i.p. route 30 min after induction of ischemia. Vehicle was administered (2 ml/kg i.p.) 30 min after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Lipoic acid (Lipo) treatment (100 mg/kg i.p.) after 30 min of ischemia induction.

3.2.1.1.3 Co-enzyme Q 10 (CoQ) alone treatment:

Co-enzyme Q10 was dissolved in pea nut oil (vehicle) and administered by i.p. route 30 min after induction of ischemia. Vehicle was administered (2 ml/kg i.p.) 30 min after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg i.p.)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Co-enzyme Q10 (CoQ) treatment (100 mg/kg i.p.) after 30 min of ischemia induction.

3.2.1.1.4 *Lagerstroemia speciosa L* (Banaba) alone treatment:

Banaba extract (dry powder) was dissolved in saline with 0.5% poly ethylene glycol (PEG as a vehicle) and administered by i.p. route 30 min after induction of ischemia. Vehicle was administered (2 ml/kg i.p.) 30 min after induction of ischemia. Following groups were involved in the study.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg i.p.)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Banaba (BNA) treatment (100 mg/kg i.p.) after 30 min of ischemia induction.

3.2.1.1.5 Progesterone + Lipoic acid (PG+Lipo) treatment:

Co-administration of progesterone and lipoic acid was carried out after 30 min of ischemia induction. Following groups were involved in the study. Vehicle was administered (2 ml/kg i.p.) 30 min after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Progesterone (2 mg/kg) + Lipoic acid (100 mg/kg i.p.) treatment after 30 min of ischemia induction.

3.2.1.1.6 Progesterone + Co-enzyme Q 10 (PG+ CoQ) treatment:

Co-administration of progesterone and Co-enzyme Q 10 was carried out as acute treatment after 30 min of ischemia induction. Vehicle was administered (2 ml/kg i.p.) 30 min after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Received progesterone (2 mg/kg) + Co-enzyme Q 10 (100 mg/kg i.p.) treatment after 30 min of ischemia induction.

3.2.1.1.7 Progesterone + Banaba (PG+BNA) treatment: Co-administration of progesterone and Banaba was carried out as acute treatment after 30 min of ischemia induction. Vehicle was administered (2 ml/kg i.p.) 30 min after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Progesterone (2 mg/kg) + Banaba (100 mg/kg i.p.) treatment after 30 min of ischemia induction.

3.2.1.1.8 Gender specific effect of PG:

The study was designed to investigate the effect of progesterone (PG 2 mg/kg i.p.) and its gender based variation on myocardial ischemia/reperfusion (I/R) injury in rats. The study involved seven groups. A Sham-operated (SO) control group and six ischemic groups. The ischemic groups include a vehicle treated I/R injury control of male (I/R-M), female (I/R-F) and ovariectomised (I/R-OVR) animals. Progesterone (PG 2 mg/kg i.p.) was administered to the animals 30 min after ischemia induction to male, female and OVR animal (I/R-M+PG, I/R-F+PG, I/R-OVR+PG) groups, respectively. Pea nut oil was used as a vehicle and administered by i.p. route.

3.2.1.1.9 Estrogen (Estro) alone treatment:

Estrogen activity was evaluated for ischemia/reperfusion injury using ovariectomized rats. Estrogen i.e.17 β-estradiol (8 µg/kg s.c.) was administered 24 h before induction of ischemia. Pea nut oil was used as a vehicle for estrogen administration.

3.2.1.1.10 Estrogen + Lipoic acid (Estro + Lipo) treatment:

Estrogen (8 µg/kg s.c.) was administered 24 h before induction of ischemia and followed by Lipoic acid (100 mg/kg i.p.)after 30 min of ischemia induction in ovariectomised animals as acute treatment. Here vehicle was administered to the

reperfusion (I/R control) injury control group twice i.e. 24 h before and 30 min after induction of ischemia. Following groups were involved in the study.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Estrogen (8 µg/kg s.c.) + Lipoic acid (100 mg/kg i.p.) treatment before 24 h and 30 min of ischemia induction respectively.

3.2.1.1.11 Estrogen + Co-enzyme Q 10 (Estro + CoQ) treatment:

Co-administration of estrogen and Co-enzyme Q 10 was carried out in ovariectomised animals as acute treatment. Estrogen was injected 24 h before ischemia induction and Co-enzyme Q 10 was dosed after 30 min of ischemia induction. Here vehicle was administered to the Reperfusion (I/R control) injury control group twice i.e. 24 h before and 30 min after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Estrogen (8 µg/kg s.c.) + Co-enzyme Q 10 (100 mg/kg i.p.) treatment before 24 h and 30 min of ischemia induction respectively.

3.2.1.1.12 Estrogen + Banaba (Estro+BNA) treatment:

Before 24 h of ischemia induction, estrogen was administered by s.c. route which was followed by administration of Banaba 30 min after ischemia induction in ovariectomised animals as acute treatment. Here vehicle was administered to the reperfusion (I/R control) injury control group twice i.e. 24 h before and 30 min after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Estrogen (8 µg/kg s.c.) + Banaba (100 mg/kg i.p.) treatment before 24 h and 30 min of ischemia induction respectively.

3.2.2 Middle cerebral artery occlusion (MCAO) induced cerebral ischemia/reperfusion (I/R) injury

Middle cerebral artery occlusion was performed to produce focal cerebral ischemia as reported by Longa et al (1989)[358] with few modifications. Male Sprague-Dawley rats, weighing 280-320 g were anesthetized with chloral hydrate (350 mg/kg i.p.), which acts as a short acting anesthetic agent and allows the animals to recover within 2 h. The body temperature was maintained at 37±1 $^{\circ}$C during surgery and ischemic period. A

midline incision was made on the ventral side of the neck to expose the left common carotid artery. After the identification of internal and external carotid artery junction, the external carotid artery was ligated and dissected. A 3-0 (Ethilon) nylon monofilament tip which was blunted by using sandpaper and coated with 0.01% poly-L-Lysine (Sigma-Aldrich) was introduced through the external carotid artery (ECA). It was advanced into the internal carotid artery (ICA) to be placed at the origin of anterior cerebral artery, which was confirmed by the slight resistance. The total length of the filament was 19-21 mm inside the ICA. The incision was sutured and the monofilament was allowed to remain in place for period of 2 h. After the ischemic period of 2 h, the filament was completely pulled out under mild anesthesia for the production of reperfusion up to 22 h. Animals were allowed to regain consciousness after the surgery period and the closure of incision. All the rats were sacrificed after 22 h of reperfusion and the brains were isolated for estimation of various parameters.

The experiment was performed in four sets of animals (n=6) for every group. The first set was used for quantification of neurological deficit, observation of physiological parameters and measurement of infarct volume. Second set was utilized for estimation of oxidative stress parameters (SOD, GSH, catalase, lipid peroxidation) and measurement of MPO activity. The effect on BBB permeability was evaluated in third set of six animals and the fourth set was employed for DNA fragmentation analysis and histological observations.

Protocol -1

Animals in the I/R control and treatment groups were evaluated for neurological deficit after 22 h of reperfusion. Neurological deficit observations were scored according to the five point scale[358]. No neurological deficit = 0, failure to extend right paw fully = 1, circling to right = 2, falling to right = 3, unable to walk spontaneously and depressed levels of consciousness = 4. The animals, which showed neurological deficit score below 3 after 2 h of ischemia, were excluded from the study. Six animals were excluded from study for unsatisfactory score. Animals were observed for exclusion criteria 2 h after ischemia and the final neurological score was evaluated only after the completion of experimental protocol of 2 h ischemia followed by 22 h of reperfusion.

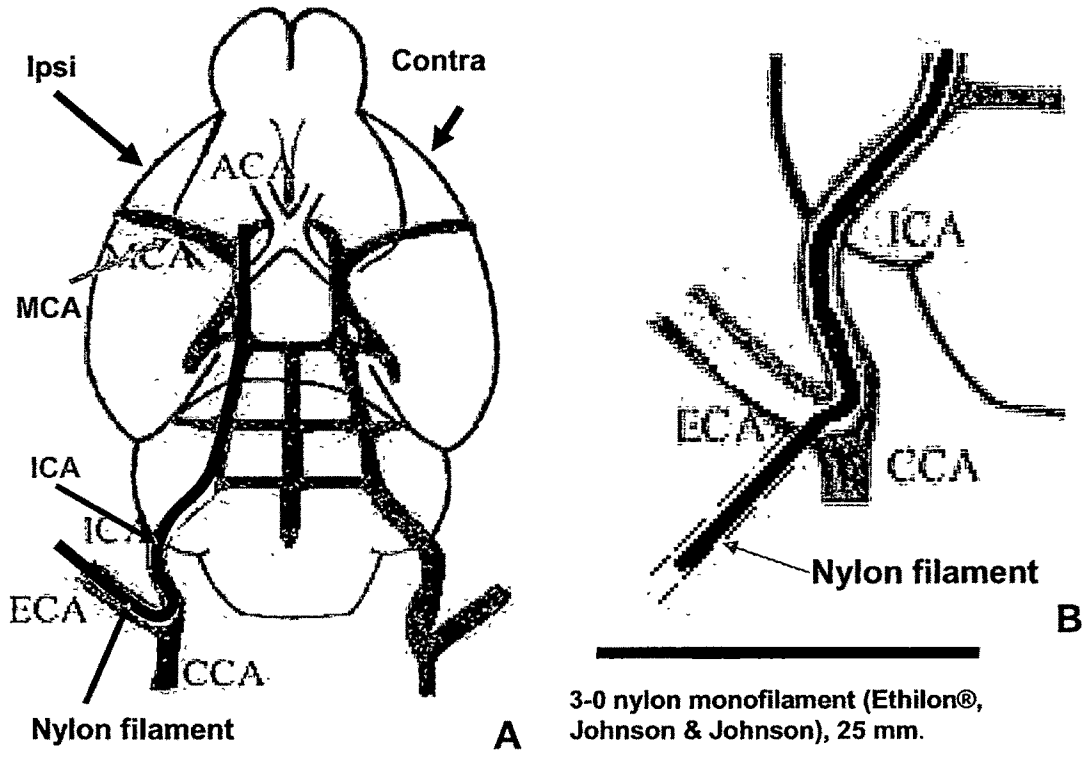

Fig. 6 Diagrammatic representation of **(A)** MCA blocked by intra-luminal thread, **(B)** ECA was pulled to form a nick for easy insertion of thread.
Ipsi Hemi = Ipsilateral hemisphere and Contra Hemi = Contralateral Hemisphere

For measurement of infarct area, the brain was promptly isolated and coronal slices with 2 mm thickness were cut and stained with 2% of 2,3,5 triphenyltetrazolium chloride (TTC) for 30 min at room temperature. Slices were fixed in 10% formalin overnight and scanned. Each slice was evaluated for infarct area using Image J software 1.30 V (rsb.info.nih/ij.). Areas of infarct of all the sections were cumulated to obtain total infarct area which was multiplied by the thickness of the brain slices to get infarct volume per brain (mm^3) and corrected for edema[33].

Brain swelling was determined as follows:

[(volume of ipsilateral hemisphere- volume of contralateral hemisphere)/volume of contralateral hemisphere(x100%)][359].

Protocol -2

Antioxidant parameters for estimations of various markers and the MPO activity in brain were carried out as described in section **3.4.2** and **3.4.5** respectively.

Protocol -3

The effect on BBB permeability was evaluated performed as described in section **3.5.**

Protocol -4

DNA fragmentation analysis and histological observations were performed as described in section **3.4** and **3.6.**

Exclusion criteria

The animals which showed neurological deficit score below 3 after 2 h of ischemia were excluded from the study. Six animals were excluded from study for unsatisfactory score. Animals were observed for exclusion criteria 2 h after ischemia.

3.2.2.1 Groups and acute treatment schedule for drug administration

Activity of progesterone and estrogen was studied on treated animals. These hormones were studied individually and in combination with various antioxidants to observe its synergistic activity if any on cerebral reperfusion injury. Effect of estrogen was observed on ovariectomised animals. Sham- operated control group (the filament was only introduced in ICA and removed immediately without blocking MCA) animals received vehicle (2 ml/kg i.p.). The vehicle treated reperfusion injury (I/R control) group was studied to observe the effect of vehicle in the study. The drugs were administered individually or in combination to study its effect on reperfusion injury.

The animals subjected to reperfusion injury received vehicle (2 ml/kg i.p.) 1 h after induction of ischemia and were considered as vehicle treated reperfusion injury control (I/R control) group

3.2.2.1.1 Progesterone (PG) alone treatment:

Progesterone was dissolved in pea nut oil (vehicle) and administered by i.p. route 1 h after ischemia induction by MCAO. Vehicle treated group was administered with vehicle (2 ml/kg i.p.) 1 h after induction of ischemia as considered as treated reperfusion injury control (I/R control) group.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg i.p.)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Progesterone (PG) treatment (2 mg/kg i.p.) after 1 h of ischemia induction.

3.2.2.1.2 Lipoic acid (Lipo) alone treatment:

Lipoic acid was dissolved in pea nut oil (vehicle) and administered by i.p. route 1 h after ischemia induction by MCAO. Vehicle treated group was administered with

vehicle (2 ml/kg i.p.) 1 h after induction of ischemia as considered as treated reperfusion injury control (I/R control) group.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Lipoic acid (Lipo) treatment (100 mg/kg i.p.) after 1 h of ischemia induction.

3.2.2.1.3 Co-enzyme Q 10 (CoQ) alone treatment:

Co-enzyme Q10 was dissolved in pea nut oil (vehicle) and administered by i.p. route 1 h after ischemia induction by MCAO. Vehicle treated group was administered with vehicle (2 ml/kg i.p.) 1 h after induction of ischemia as considered as treated reperfusion injury control (I/R control) group.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg i.p.)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Co-enzyme Q10 (CoQ) treatment (100 mg/kg i.p.) after 1 h of ischemia induction.

3.2.2.1.4 Lagerstroemia speciosa L (Banaba) alone treatment:

Banaba extract (dry powder) was dissolved in saline with 0.5% PEG (vehicle) and administered by i.p. route 1 h after induction of ischemia. Following groups were involved in the study. Vehicle treated group was administered with vehicle (2 ml/kg i.p.) 1 h after induction of ischemia as considered as reperfusion injury control (I/R control) group.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg i.p.)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Banaba (BNA) treatment (100 mg/kg i.p.) after 1 h of ischemia induction.

3.2.2.1.5 Trimetazidine (TMZ) alone and its combination with PG on cerebral I/R injury:

Trimeatzidine was dissolved in saline (vehicle) and administered by i.p. route in two doses 1 h after induction of ischemia. Following groups were involved in the study.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg i.p.)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Received TMZ (5mg/kg i.p.) after 1 h of ischemia induction.

Group III: Received TMZ (25mg/kg i.p.) after 1 h of ischemia induction.

Group IV: Combination of PG (2 mg/kg i.p.) and TMZ (25 mg/kg i.p.) was administered to the animals.

3.2.2.1.6 Progesterone + Lipoic acid (PG+Lipo) treatment:

Progesterone (2 mg/kg i.p) and lipoic acid was co-administered as acute treatment after 1 h of ischemia induction. Following groups were involved in the study.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Progesterone (2 mg/kg) + Lipoic acid (100 mg/kg i.p.) treated 1 h of ischemia induction.

3.2.2.1.7 Progesterone + Co-enzyme Q 10 (PG+ CoQ) treatment:

Co-administration of progesterone and Co-enzyme Q 10 was carried out as acute treatment after 1 h of ischemia induction. Following groups were involved in the study.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Progesterone (2 mg/kg) + Co-enzyme Q 10 (100 mg/kg i.p.) treatment after 1 h of ischemia induction.

3.2.2.1.8 Progesterone + Banaba (PG+BNA) treatment:

Progesterone was administered by i.p. route 1 h after ischemia induction which was immediately followed by administration of Banaba by i.p. route. Following groups were involved in the study.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Progesterone (2 mg/kg) + Banaba (100 mg/kg i.p.) treatment after 1 h of ischemia induction.

3.2.2.1.9 Estrogen (Estro) alone treatment:

Estrogen activity was evaluated for cerebral ischemia/reperfusion injury using ovariectomized rats. Estrogen i.e.17 β-estradiol (8 µg/kg s.c.) was administered 24 h before induction of ischemia by MCAO. Estrogen was dissolved in pea nut oil.

3.2.2.1.10 Estrogen + Lipoic acid (Estro + Lipo) treatment:

Estrogen (8 µg/kg s.c.) was administered 24 h before induction of ischemia and followed by Lipoic acid (100 mg/kg i.p.) after 1 h of ischemia induction by MCAO in ovariectomised animals as acute treatment. Vehicle was administered to the Reperfusion (I/R control) injury control group twice i.e. 24 h before and 1 h after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Received Estrogen (8 µg/kg s.c.) + Lipoic acid (100 mg/kg i.p.) treatment before 24 and 1 h of ischemia induction respectively.

3.2.2.1.11 Estrogen + Co-enzyme Q 10 (Estro + CoQ) treatment:

Co-administration of estrogen and Co-enzyme Q 10 was carried out in ovariectomised animals as acute treatment. Estrogen was injected 24 h before ischemia induction and Co-enzyme Q 10 was dosed 1 h after ischemia induction. Vehicle was administered to the Reperfusion (I/R control) injury control group twice i.e. 24 h before and 1 h after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Received Estrogen (8 µg/kg s.c.) + Co-enzyme Q 10 (100 mg/kg i.p.) treatment before 24 and after 1 h of ischemia induction respectively.

3.2.2.1.12 Estrogen + Banaba (Estro+BNA) treatment:

Estrogen was injected (s.c.) to the ovariectomised animals 24 h before induction of ischemia and this was followed by intraperitonial administration of Banaba (1 h after ischemia induction) as acute treatment. Vehicle was administered to the Reperfusion (I/R control) injury control group twice i.e. 24 h before and 1 h after induction of ischemia.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Received Estrogen (8 µg/kg s.c.) + Banaba (100 mg/kg i.p.) treatment before 24 and 1 h after induction of ischemia respectively.

3.2.2.1.13 Progesterone + Trimetazidine (PG+TMZ-25) treatment:

Progesterone (2 mg/kg i.p) and Trimetazidine (25 mg/kg i.p.) was co-administered as acute treatment after 1 h of ischemia induction. Following groups were involved in the study.

Group I: Sham operated (SO) control animals treated with vehicle (2 ml/kg)

Group II: Vehicle treated reperfusion injury control (I/R control) group (2 ml/kg i.p.)

Group III: Progesterone (2 mg/kg) + Trimetazidine (25 mg/kg i.p.) treated 1 h of ischemia induction.

The above mentioned groups of animals were subjected to I/R injury by the LCA and MCAO models and the parameters studied were as follows,

3. 3. Hemodynamic and ECG parameters observations

The animals which are subjected to myocardial reperfusion injury were studied for hemodynamic and ECG parameters.

3. 3. 1 Cannulation of Carotid artery and measurement of Hemodynamic parameters

The animal was anaesthetized and a lateral incision was given on the neck region. The carotid artery was traced and clamped using a sterilized bull dog clamp. A small catheter was introduced in the carotid artery and it was cannulated for the measurement of hemodynamic parameters using transducer (SL 13L, Biopac Systems) (Shinghala and Balaraman, 2005). The hemodynamic parameters observed are systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MAP). All the data were analyzed using Biopac Student Lab Pro software (Version 3.6.7).

3. 3. 2 Measurement of Electrocardiographic parameters

After anaesthetizing the animal, metal probes were attached to the limbs of the animal in position Lead II. After stabilization period of 15 min., the changes in ST elevation, ST-segment prolongation were measured by ECG pattern evaluation (SS2L, Biopac System) at intervals before ischemia (Baseline), at the end of ischemic period (End-ischemia) and after the completion of reperfusion period (End-reperfusion).

3. 4. Biochemical and Biomarker Estimations

3. 4. 1 Collection and Processing of Serum and Tissues for Estimations

Serum:

At the end of the treatment period, rats were anaesthetised with anesthetic ether. Blood was collected from the retro-orbital plexus without any anti-coagulant and allowed to clot for 10 minutes at room temperature. It was then centrifuged at 2500 rpm for 20 minutes. The serum obtained was stored at 4°C till further use.

Collection of Tissues Homogenate:

Reagents

1. Phosphate Buffered Saline pH 7.4-

Disodium ethylenediaminetetraacetic acid (1.38 gm), 0.19 gm of potassium dihydrogen phosphate and 8 gm of sodium chloride was dissolved in 900 ml of distilled

water and adjusted pH using dilute hydrochloric acid. The volume was adjusted to 1000 ml using distilled water.

1. Sucrose solution (0.25M)

Sucrose (85.58gm) was dissolved in 200 ml of water and diluted to 1000 ml with distilled water.

2. Tris hydrochloride buffer (10mM, pH 7.4)

Tris buffer (1.21gm) was dissolved in 900 ml of distilled water and the pH was adjusted to 7.4 with 1M hydrochloric acid. The resulting solution was diluted to 1000 ml with distilled water.

Procedure

After prompt decapitation of animal, heart or brain was isolated and quickly transferred to ice-cold phosphate buffered saline (pH 7.4). It was blotted free of blood and tissue fluids, weighed on a Single Pan Electronic Balance (Precisa 205 ASCS). The heart or brain was cross-chopped with surgical scalpel into fine slices, suspended in chilled 0.25M sucrose solution and quickly blotted on a filter paper. In case of brain ipsilateral and contralateral hemispheres were separated. The tissues were then minced and homogenised in chilled tris hydrochloride buffer (10mM, pH 7.4) to a concentration of 10% w/v. Prolonged homogenisation under hypotonic condition was designed to disrupt, as far as possible, the structure of the cells so as to release soluble proteins. The homogenate was centrifuged at 7,000 rpm at 0°C for 25 minutes using Remi C-24 high speed cooling centrifuge. The clear supernatant was used for the determination of superoxide dismutase and catalase. The sediment was re-suspended in ice-cold tris hydrochloride buffer (10mM, pH 7.4) to get a final concentration of 10% w/v and was used for the estimation proteins.

3. 4. 2 Biomarkers of oxidative stress in tissue-

The oxidative stress parameters were estimated by measuring the biomarkers in tissue (heart and brain) samples. The tissue levels of lipid peroxidation (MDA content), Superoxide Dismutase (SOD) Catalase (CAT) and Reduced Glutathione (GSH) were estimated as biomarkers of oxidative stress.

3. 4. 2 .1 Determination of Lipid Peroxidation (MDA content)

It was estimated using the method described by Slater and Sawyer (1971)[360] which is as follows.

Reagents

1. *Thiobarbituric acid (TBA)(0.67% w/v)*

   Thiobarbituric acid (0.67gm) was dissolved in 50 ml of hot distilled water and the final volume was made up to 100 ml with hot distilled water.

2. *Trichloroacetic acid (TCA) (20% w/v)*

   Trichloroacetic acid (20gm) was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

3. *Standard Malondialdehyde (MDA) stock solution (50mM)*

   A standard malondialdehyde stock solution was prepared by mixing 25µl of 1, 1, 3, 3-tetraethoxypropane up to 100 ml with distilled water. 1.0 ml of this stock solution was diluted up to 10 ml to get solution containing 23µg of malondialdehyde/ml. One ml of this stock solution was diluted up to 100 ml to get a working standard solution containing 23ng of malondialdehyde/ml.

Procedure

a) Add supernatant of the tissue homogenate (2 ml) and TCA 20% (2 ml)
b) Allow to stand in ice bath for 15 min
c) Centrifuge the mixture
d) Add 2 ml of TBA to 2 ml of clear supernatant
e) Heat in boiling water bath for 15 min and cool in ice bath for 5 min
f) Colour developed and was measure at 532 nm against blank.

Different concentrations (0-23nM) of standard malondialdehyde were taken and processed as above for standard graph. The values were expressed as nM of MDA/mg protein.

3. 4. 2 .2 Superoxide Dismutase (SOD)

Superoxide dismutase was estimated using the method developed by Misra and Fridovich (1972)[361] which is as follows.

Reagents

1. Carbonate Buffer (0.05M, pH 10.2)

Sodium bicarbonate (16.8gm) and 22gms of sodium carbonate was dissolved in 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.

2. Ethylenediaminetetra acetic acid (EDTA) solution (0.49M)

EDTA (1.82gm) was dissolved in 200 ml of distilled water and the volume was made up to 1000 ml with distilled water.

3. Hydrochloric acid (0.1N)

Concentrated hydrochloric acid (8.5 ml) was mixed with 500 ml of distilled water and the volume was made up to 1000 ml with distilled water.

4. Epinephrine solution (3mM)

Epinephrine bitartarate (0.99gm) was dissolved in 100 ml of 0.1N hydrochloric acid and the volume was adjusted to 1000 ml with 0.1N hydrochloric acid.

5. Superoxide Dismutase (SOD) standard (100 U/L)

SOD (1mg i.e.1000 U/mg) from bovine liver was dissolved in 100 ml of carbonate buffer.

Procedure

a) Dilute 0.5 ml supernatant of the tissue homogenate with distilled water
b) Add 0.25 ml of ethanol and 0.15 ml of chloroform (ice cold)
c) Centrifuge the mixture at 2500 rpm for 5 min
d) Add 1.5 ml of carbonate buffer, 0.5 ml of EDTA to 0.5 ml of supernatant
e) Reaction was initiated by addition of 0.4 ml of Epinephrine
f) Change in optical density/ min was measure at 480 nm.

SOD activity was expressed as units/mg protein. Change in optical density per minute at 50% inhibition of epinephrine to adrenochrome transition by the enzyme is taken as the enzyme unit. Calibration curve was prepared by using 10-125 units of SOD.

3. 4. 2. 3 Catalase (CAT)

Catalase estimated by the method of Hugo Aebi as given by Colowick et al. (1984)[362] which is as follows.

Reagents

1. Phosphate Buffer (50mM/l, pH 7.0)

(a) 6.81gms of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in distilled water and made up to 1000 ml.

(b) 8.90gms of disodium hydrogen orthophosphate (Na_2HPO_4) was dissolved in distilled water and made up to 1000 ml.

The solutions (a) and (b) were mixed in the proportion of 1: 1.5 (v/v).

Hydrogen Peroxide (H_2O_2) (30 mmol/l)

0.34ml of 30% hydrogen peroxide was diluted with phosphate buffer to 100 ml. This solution was prepared fresh each day.

4. Catalase standard (65,000 U/mg protein; 1mg protein/ml)

Crystalline beef-liver catalase suspension was centrifuged to isolate the crystals of the enzyme that was dissolved in 0.01M phosphate buffer (pH 7.0) to give a final concentration of 1.0mg protein/ml. Before assay, it was diluted with distilled water to obtain 1000 U/ml.

Procedure

a) Dilute supernatant of the tissue homogenate (aprrox 20 times) with Phosphate buffer. Same dilution with Blank as wel.

b) Add 1ml of hydrogen peroxide (30 mM/l) to initiate reaction

c) Dilution should be such that the initial absorbance should be approx 0.500.

d) Change in absorbance was measured at 240 nm.

Catalase activity was expressed as μmoles of H_2O_2 consumed/min/mg protein.

3. 4. 2 .4 Reduced Glutathione (GSH)

Reduced glutathione was determined by the method described by Moron et al. (1979)[363] which is as follows.

Reagents

1. Trichloroacetic acid (10% w/v)

Trichloroacetic acid (10gm) was dissolved in sufficient quantity of distilled water and the final volume was made up to 100 ml with distilled water.

2. Phosphate Buffer (0.2M, pH 8.0)

Sodium phosphate (0.2M) was prepared by dissolving 30.2gms sodium phosphate in 600 ml of distilled water, the pH was adjusted to 8.0 with 0.2M sodium hydroxide solution and the final volume was adjusted up to 1000 ml with distilled water.

3. DTNB reagent (0.6mM)

60mg of 5, 5'-dithiobis (2-nitro benzoic acid) was dissolved in 50 ml of buffer and the final volume was adjusted to 100 ml with buffer.

4. Standard Glutathione

Reduced glutathione (10mg) was dissolved in 60 ml of distilled water and the final volume was made up to 100 ml with distilled water.

Procedure

a) Equal volume of supernatant and 20% TCA were mixed.

b) The precipitated fraction was centrifuged

c) Supernatant (0.25 ml) and 2 ml of DTNB were added.

d) Final volume of 3 ml was made up with phosphate buffer.

e) Change in colour development was measured at 412 nm.

Different concentrations (10-50μg) of standard glutathione were taken and processed as above for standard graph.

The amount of reduced glutathione was expressed as μg of GSH/mg protein.

3. 4. 3. Estimation of Total Protein

The method of Lowry et al. (1951)[364] was used for the estimation of total protein which is as follows.

Reagents

1. Sodium hydroxide (0.1M) —

Sodium hydroxide (4 mg) was dissolved in 400 ml of distilled water and the final volume was made up to 1000 ml with distilled water.

2. Lowry C reagent

a) Copper sulphate in 1% sodium potassium tartarate (1% w/v) 0.5gm of copper sulphate was dissolved in 1% sodium potassium tartarate (Prepared by dissolving 1gm of sodium potassium tartarate in 100 ml of distilled water).

b) Sodium carbonate in 0.1M sodium hydroxide (2% w/v)
2gms of sodium carbonate was dissolved in 100 ml of 0.1M sodium hydroxide.

2ml of solution (a) was mixed with 100 ml of solution (b) just before use.

3. Standard Protein (Bovine serum albumin)

20mg of bovine serum albumin was dissolved in 80 ml of distilled water and few drops of sodium hydroxide were added to aid complete dissolution of bovine serum albumin and to avoid frothing. Final volume was made up to 100 ml with distilled water and stored overnight in a refrigerator.

4. Folin's phenol reagent

Folin's phenol reagent was diluted with distilled water in the ratio of 1:2. (i.e. 1ml of Folin's phenol reagent was mixed with 2ml of distilled water).

Procedure

a) Diluted membrane fraction aliquots (0.1ml) were taken.

b) Add 0.8ml of 0.1M sodium hydroxide and 5ml of Lowry C and allowed to stand for 15 min.

c) Add 0.5ml of 1N Folin's phenol reagent and mix well.

d) Absorbance measured at 640 nm.

Different concentrations (40-200µg) of standard protein (Bovine serum albumin) were taken and processed as above for standard graph.

The values were expressed as mg of protein/ gm of wet tissue (mg/gm).

3.4.4 Serum biomarkers of myocardial injury Parameters

Creatine kinase (CK)

Quantitative estimation of creatine kinase was performed as per instructions provided for ENZOPAK-CK-NAC kit using RA 50 auto analyser. Units were expressed as U/l.

3.4.5 Measurement of Myeloperoxidase (MPO) activity

MPO was extracted and the activity was measured using a modified spectroscopic method described by Bradley et al (1982) which is as follows.

Reagents

1) Potassium phosphate buffer (50mM pH 6.0)

Dissolve 3.402gm KH_2PO_4 in 450ml, adjust to pH 6.0, make up to 500ml.

2) Extraction Buffer

Dissolve 0.5gm HTAB (hexadecyltrimethylammonium bromide) in 100ml phosphate buffer.

3) Substrate solution (0.167mg/ml dianisidine)

Dissolve 16.7mg o-dianisidine in 100ml phosphate buffer. If there is a precipitate in solution filter using 0.2µm filter. Cover in alfoil as this substrate is light sensitive and store at 4°C. Solution lasts approximately 1 week. Discard if solution becomes slightly brownish. On the day of the assay, warm solution up to

room temperature and add hydrogen peroxide (100µl of 0.5% per 100ml substrate solution) approx 30 mins before use.

Procedure

a) Tissue samples (heart or brain) were homogenized in ice cold 50mM potassium phosphate buffer pH-6 containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB, Sigma).

b) The homogenate was freeze thawed three times then centrifuged at 11000 x g for 20 min at 4°C.

c) The supernatant (34 µl) was mixed with the same phosphate buffer (986 µl) containing 0.167 mg/ml ortho-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide .

d) The change in absorbance at 460 nm was recorded by spectrophotometer.

e) One unit of MPO activity was defined as that consuming 1nanomole of peroxide per minute at 22° C.

The results were expressed as milliunit/ (mg of protein)

3.5 DNA fragmentation Analysis by Gel Electrophoresis

DNA fragmentation analysis was performed using method given by Simonian et al (1996)[366].

Reagent

1) Tris-Hcl (100mM, pH 8.0)

Dissolve 1.21 gm in 100 ml of distilled water.

2) EDTA (5mM/l)

Dissolve 186 mg of EDTA in distilled water.

3) SDS (0.2%)

Add 200 mg of SDS in 100 ml of warm distilled water.

4) RNase A

Add 5 mg of RNase A in to 1 ml of Tris- Hcl buffer.

Procedure

a) The heart or the cortex of ipsilateral hemisphere of brain after reperfusion was used to harvest the DNA.

b) Tissue samples were immediately digested in 100 mM/l Tris-Hcl (pH 8.0), 200 mM/l NaCl, 5 mM/l EDTA and 0.2% SDS with 5 mg/ml RNase A.

c) Then extracted using phenol-chloroform.

d) Approximately 8 μg of DNA was loaded in each lane and run at 70 V on a 0.8% agarose gel and stained with ethidium bromide (0.5 μg/ml) to separate DNA.

e) A 1K DNA (Gene Ruler 1K Fermentas, Germany) ladder was used as a reference standard.

Since extensive DNA fragmentation is an important characteristic of apoptosis, visualization of DNA breaks could greatly facilitate the identification of apoptotic cells.

3.6 Quantification of BBB by Evans Blue (EB) Extravasation

Measurement EB extravasation was used to evaluate the blood-brain barrier permeability[367].

Reagent

1) Evans Blue (4%)

Dissolve 80mg of Evans Blue in 2 ml saline solution.

2) Heaprinised saline solution

Add 1000 IU heparin to saline solution

3) Potassium phosphate buffer (0.1 M)

Dissolve 238 mg of Na_2HPO_4, 19 mg of KH_2PO_4 and 800 mg of NaCl in 100 ml of distilled water.

4) Trichloroacetic acid (100%)

Procedure

a) At the commencement of reperfusion, EB (Sigma-Aldrich, 0.1 ml of 4% solution) was injected into the tail vein.

b) Rats were transcardially perfused with 100 ml of heparinized saline solution (10 IU/ml) 22 h after reperfusion.

c) Brain was removed and ipsilateral and contralateral hemispheres were separated. Each hemisphere was homogenized in 1 ml of 0.1 M PBS and then centrifuged at 1000 x g for 15 min.

d) Trichloroacetic acid (0.7 ml of 100%) was added into 0.7 ml of supernatant. The mixture was allowed to incubate at 14°C for 18 h and then centrifuged at 1000 x g for 30 min.

e) The amount of EB in supernatant was spectrophotometrically measured at 610 nm wavelength by comparison with readings obtained from standard solutions.

Blood brain permeability was quantified as micrograms of EB per hemisphere

3.7 Histopathological Studies

After the experimental period as finalized for either of model was over, the animals were sacrificed and the heart and brain were excised, blotted free of blood and tissue fluids and preserved in 10% v/v formal saline solution. The specimens were sectioned, stained and mounted for the detail histopathological study. Briefly, after a week the tissues were washed thoroughly in repeated changes of 70% alcohol and then dehydrated in ascending grades of alcohol (70-100%). After dehydration, the tissues were cleaned in xylene and embedded in paraffin wax. Sections of 5μ thickness were cut on a microtome and taken on glass slides coated with albumin. The sections were deparaffinated in xylene and downgraded through 100, 90, 70, 50, 30% alcohol and then finally in water. The haematoxylin-stained sections were stained with eosin for 2 minutes and were then quickly passed through ascending grades of alcohol, cleaned in xylene and mounted on Canada balsam. The stained sections were examined under Olympus BX40 Photomicroscope and photographed. Either samples were coded to perform blind study or expert guidance from the veteran pathologist was sought to determine histopathological changes.

3.8 Statistical Analysis of Data

All the result of biochemical estimations have been indicated in terms of mean ± SEM. Difference between the groups was statistically determined by analysis of variance (ANOVA) with Tukey post comparison test using GraphPad InStat version 3.00, GraphPad Software, California USA. The level of significance was set at $p < 0.05$.