CHAPTER 7

PRE-ADMINISTRATION OF AMLODIPINE AND METOPROLOL COUPLED WITH BILATERAL COMMON CAROTID ARTERY OCCLUSION INDUCES BRAIN LESIONS IN RATS

Highlights of the chapter

- *The amlodipine and metoprolol can induce hypotension and reduced cerebral blood flow after 30 minutes of administration in rats.*
- *The results demonstrated that 1h of BCCAO could induce ischemia in the rat brain.*
- *Hypotension coupled with BCCAO increased BBB permeability, produced larger infarction volume and characterized by higher number of apoptotic and necrotic cells compared to BCCAO alone*

Abstract

Study of global cerebral ischemia demands simple and reproducible rodent models with fewer chances of mortality. 2-vessel and 4-vessel occlusion models are commonly used for induction of global cerebral ischemia, but the surgical complexity and high mortality rate associated with the 4-vessel occlusion model makes it an unpopular model of choice for laboratory experiments. The 2-vessel occlusion (2VO) or bilateral common carotid artery occlusion (BCCAO) model is relatively simple and reproducible to induce global cerebral ischemia, but it has spurred controversies. The 2VO with hypotension is a widely accepted model that produces marked forebrain lesions and used for the evaluation of neuroprotective agents. This model requires lowering of blood pressure by withdrawing blood from a femoral artery or jugular vein which makes it complicated. Therefore, the present study describes a model that use vasodilator drugs (amlodipine and metoprolol) to induce the hypotension followed by BCCAO for investigating the cerebral infarction, blood-brain barrier (BBB) disruption, histological changes as well as brain cell death in rats. The study also analyzes the changes in

physiological parameters like regional cerebral blood flow, systemic blood pressure and heart rate with and without administration of vasodilator drugs. Somewhat unexpectedly, a large cerebral infarction, an increased BBB permeability, marked histological changes as well as higher number of apoptotic and necrotic cells were observed in rats subjected to hypotension followed by BCCAO as compared to that of rats subjected to BCCAO alone. In conclusion, we can say that this model is suitable to study the biochemical and molecular parameters and for evaluation of neuroprotective procedures.

7.1 INTRODUCTION

Focal and global cerebral ischemia is the most frequently used models for studying ischemic brain conditions in animals [271]. Global or forebrain ischemic models include 2-vessel occlusion (2VO or BCCAO) and 4-vessel occlusion (4VO), which are suitable for studying neuronal damage induced molecular alterations in cerebral ischemia-reperfusion injury [36]. Physiologically controlled, reproducible ischemic stroke models are essential for the investigation of molecular mechanisms, therapeutic procedures, and prevention of ischemic insult. Therefore, rodents have mostly opted for cerebral ischemic models since they are easy to handle, comparatively cheaper, relatively homogenous inbred strains and simple surgical methods are involved [272]. The 2VO model involves occlusion of both CCAs to induce global cerebral ischemia, but the method has remained controversial. Several studies has been demonstrated that BCCAO can induce ischemia and cause neuronal damage [92],[93],[94] [95],[96]. In contrast to the studies mentioned above, it was recently suggested that acute BCCAO alone might not be a suitable strategy to induce ischemic condition in rats [38]. Moreover, various studies suggest that 84% of patients of acute stroke are also diagnosed

with hypertension and a small number of patients have blood pressure lower than the typical value associated with cerebral ischemia. In both cases of moderate or increased blood pressure, the stroke condition generally worsens. Though the association of hypotension with transient ischemic attacks (TIAs) and stroke remains controversial, the possibility of induction of focal neurological deficits in patients with carotid artery stenosis due to hypotension was suggested by Denny-Brown and colleagues. Contrary to this, several recent studies state the TIAs or stroke is rarely caused by hypotension. However, a study by Ruff et al. shows that in hypertensive patients with hemodynamically significant carotid artery stenosis, low blood pressure occurring due to postural change, antihypertensive medications or cerebral angiography can be associated with transient focal neurological deficits. Similar conditions of cerebral ischemia have been observed in an animal model in which hypotension has been induced artificially. Smith et al. demonstrated that a systemic lowering of MAP to 40 mmHg causes reduction of perfusion through the vertebral arteries leading to highly lowered or no blood flow. The condition mentioned above when coupled with occlusion of carotid arteries produced forebrain ischemia [98]. The resulting brain damage due to this process exhibits a pattern similar to patients surviving cardiac arrest [39,98]. But, the model remains complex due to the requirement of blood withdrawal from either a femoral artery or jugular vein.

Induction of hypotension can also be achieved by administration of vasodilators. Amlodipine is a third generation dihydropyridine (DHP), calcium channel blocker (CCB) and Metoprolol is a first generation selective ß1 blocker. Both drug alone or in combination reduce the blood pressure and has been used for the treatment of hypertension (HTN)/high blood pressure (BP) and angina [273,274]. No known study has been reported for the analysis of

cerebral infarction and blood-brain barrier disruption in rats after inducing hypotension (using vasodilator drug) coupled with bilateral common carotid artery occlusion.

Therefore, the present study is designed to explore the effect of administration of amlodipine and metoprolol, followed by tBCAAO in the induction of cerebral infarction in rat brain. Also, the present study aims to design a simple and reproducible rat model for studying both acute cerebral ischemia and the associated pathophysiological changes due to brain damage.

7.2 MATERIALS AND METHODS

7.2.1 Chemicals and reagents

Revelol AM (5/50 mg) Tablets (Ipca Laboratories Pvt. Ltd., India) were purchased from a medicine shop under doctor prescription. Evans Blue and 2,3,5-Triphenyltetrazolium Chloride (TTC) were purchased from TCI Chemicals (India) Pvt. Ltd. Annexin V Apoptosis Detection Kit (sc-4252 AK) was obtained from Santa Cruz Biotechnology, Inc., USA. Ultrapure water was obtained by a Milli-QVR water purification system (Merck Life Science Private Limited, India).

7.2.2 Animals and grouping

Inbred male albino Charles Foster rats (150 ± 20 g) acclimatized under standard laboratory conditions with free access to water and food. 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). The rats were equally and randomly divided into four groups consisting of control (n=10, only received saline), positive control (n=10, received amlodipine and metoprolol 0.5/5 mg/kg in saline), BCCAO (n=10, only received saline and subjected to

ischemia) and htBCCAO (n=10; hypotension coupled with bilateral common carotid artery occlusion; received amlodipine and metoprolol 0.5/5 mg/kg 1h before induction of ischemia).

7.2.3 Hypotensive drug administration

Revelol AM (5/50 mg) each tablet contain amlodipine 5 mg and metoprolol 50 mg. One tablet of Revelol AM was crushed using mortar and pestle and dispersed in 50 ml of normal saline solution by vortexing for 15 minutes. The positive control and htBCCAO group were orally received amlodipine and metoprolol 0.5/5 mg/kg of dose and dose volume 5ml/kg by oral gavage.

7.2.4 Blood pressure and rCBF measurement

The heart rate (HR), systolic blood pressure (SBP) and rCBF of all rats were measured after anesthesia. The effect of amlodipine and metoprolol on HR, SBP, and rCBF of the positive control group were analyzed at 30, 60 and 120 minutes after oral administration of the drug. The HR and SBP of animals were measured using non-invasive blood pressure (NIBP) monitor facilitated with a specialized tail cuff and pulse transducer (IN125/R; AD Instruments Pty Ltd, Australia). The rCBF of left and right hemispheres were measured using laser Doppler blood flow (LDF) meter (INL191 Blood Flow Meter AD Instruments Pty Ltd, Australia). For the measurement of rCBF, the anesthetized rat was placed on a stereotaxic instrument (INCO, Ambala India) in the prone position and a midline incision of 2 cm in the pre-shaved scalp's skin was performed to expose the skull bone. After disinfection with povidone-iodine, the soft tissue is gently removed from the surface of the skull using a sterile cotton swaps and dental scraper. A burr hole of 1.5 mm diameter located at 5 mm lateral (left and right) and 2 mm caudal to the bregma was formed using a dental drill, by keeping the dura intact [275]. The LDF needle probe was fixed 0.5 mm above the dura surface to measure the rCBF.

7.2.5 The cerebral ischemia-reperfusion surgical procedure

The surgery was performed according to Sanderson et al. except insertion of catheter tubing into the femoral artery for blood withdrawing [39]. The animals of BCCAO and htBCCAO groups received intraperitoneal injection of anesthetic agents ketamine (50 mg/kg b.w.) and xylazine (10 mg/kg b.w.) and were placed and fixed on the surgical table in the supine position. The surgical procedure to induce global cerebral ischemia using BCCAO is discussed in section 5.2.3 of chapter 5. In this study, the artery is occluded for one hour followed by reperfusion of one hour. The control and positive control group's animals received all surgical procedures except the ligation of CCAs.

7.2.6 Estimation of cerebral infarction area

The cerebral infarction area of all groups was determined using triphenyltetrazolium chloride (TTC) staining [276]. Animals of BCCAO and htBCCAO groups were sacrificed 1 hour of occlusion followed by 1 hour of reperfusion (1/1h I/R) injury. The brains were carefully removed from the skull after sacrificed by cervical dislocation, immediately rinsed with normal saline and kept in -20°C for 5 minutes. After incubation, the brain was transferred on an ice pack, and coronal sections of 2 mm were taken in between of frontal pole and corticocerebellar junction. All brain sections were incubated at 37°C for 30 minutes in TTC (2% in saline) and were post-fixed in 10% formalin for 20 minutes. The living brain tissue is brick red whereas the infarct region appears as white. All the experiments were performed by the investigator blinded to the treatment groups. The slices were kept on the scanner glass, and color pictures (300 dpi) were taken for the further analysis. The infarct area and brain area of all slices were manually outlined, measured and quantified by NIH Image J software (NIH Image 1.52a; National Institute of Health; Bethesda, MD) as well as percent infarction were calculated [277].

7.2.7 Evans blue (EB) extravasation

The integrity of the blood-brain barrier (BBB) was investigated using Evans blue extravasation (EBE) with slight modification [278]. Before the injection of Evans blue, vasodilatation was induced in the anesthetized rats by wiping the tail by lukewarm water using a cotton swab. The Evans blue (20 mg/kg of 20 mg/ml solution) was injected intravenously in the lateral vein of the tail with a 1ml syringe during reperfusion. After 1h of EB administration, the chest was opened, and the animals were perfused through the left ventricle with 200ml of chilled saline. Further, animals were euthanized by cervical dislocation and brains were carefully removed, washed with saline and air dried after placing on labeled aluminum foil. Each brain was weighed, and coronal sections of 2 mm were taken. The sections of the brain were fixed in 10% formalin for 20 minutes, and color pictures (300 dpi) were captured using a scanner. After acquiring images, the sections were chopped in small pieces and immersed in a labeled falcon tube (wrapped with aluminum foil) containing formamide (8ml formamide/1g of dry tissue) to extract the EB out of the tissue. The tubes containing brain sample was incubated in an oven at 56ºC over two days. After incubation, blue colored formamide was transferred to a covered, labeled Eppendorf tube for the further analysis. A set of standard samples were prepared by diluting the EB in the formamide solution of eight exponential concentrations (0.1-12.8µg/mL). 200µl of the standard solutions and brain's extracted formamide samples were placed in a 96 multi-well plate, and absorbance was taken using a multi-mode reader (BioTek Instruments, Inc., USA) at a wavelength of 620 nm. The concentration of EB of each brain sample was calculated using the standard curve. The concentration of EB of each brain sample was calculated using the standard curve, multiplied by dilution factor and expressed as µg/g tissue of wet weight of brain tissue.

7.2.8 Histological investigation

The histological changes of rat brain were analyzed using Hematoxylin and Eosin Staining [279]. Briefly, the rats were subjected to transcardial perfusion with chilled saline as discussed for the EB extravasation study. Then brain was cautiously removed and immediately fixed with 10% formalin solution. The brain was embedded in paraffin, coronally sectioned at 5 μm for staining with H&E and was analyzed with a fluorescent microscope (Nikon Instruments Inc.).

7.2.9 Analysis of brain cell death

The apoptosis and necrosis of brain cells of each groups were analyzed using Annexin V Apoptosis Detection Kit procedure with some modification. Briefly, brain of anesthetized rats was cautiously removed after cervical dislocation and washed in PBS. The desired portion of brain sample (0.5gm) was taken and minced using razor blade. The tissue sample then placed in a tube containing 1ml cold Hank's Balanced Salt Solution (HBSS), 20 mM EDTA, 10% DMSO and 1ml trypsin enzyme. The tube was incubated at 37^0C for 30 minutes in a water bath for the cell dissociation. The components of the tube was mixed gently by inverting the tubes 5 times at every 10 minutes. After incubation, the sample is centrifuged at 200 RCF for 30 minutes at 4^0C. After centrifugation, the supernatant was discarded and pellet was re-suspended in 2ml HBSS as well as filtered using cell strainer of 70μm pore size. The filtered sample was further diluted to achieve cell density of 0.5-1.0 x 10^5. 100μl of sample was taken in a labelled tube and 0.5 μg of Annexin V FITC and Propidium Iodide (PI**)** was added and incubated for 15 minutes at room temperature. After incubation, 10μl of cell suspension was placed on glass slides and covered with glass coverslip and immediately analyzed under fluorescent microscope using a dual filter set for FITC and rhodamine at 40X magnification.

7.2.10 Statistical Analysis

The data were analyzed by one-way analysis of variance (ANOVA) followed by 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.

7.3 RESULTS

7.3.1 Blood pressure and Regional cerebral blood flow

First, the effect of amlodipine and metoprolol administration on systolic blood pressure, pulse rate and rCBF of control and positive control groups were studied. The mean value of systolic blood pressure, pulse rate and rCBF of normal and hypotension induced rat are presented in Fig 7.1. The SBP and pulse rate are significantly reduced ($p<0.001$) after 30, 60 and 120 minutes of drug administration compared to normal condition. rCBF of both hemisphere of the rat brain after drug administration was also significantly reduced at 60 and 120 minutes ($p<0.001$) compared to normal rat brain. However, there was no significant change observed after 30 minutes of drug administration from normal brain's rCBF.

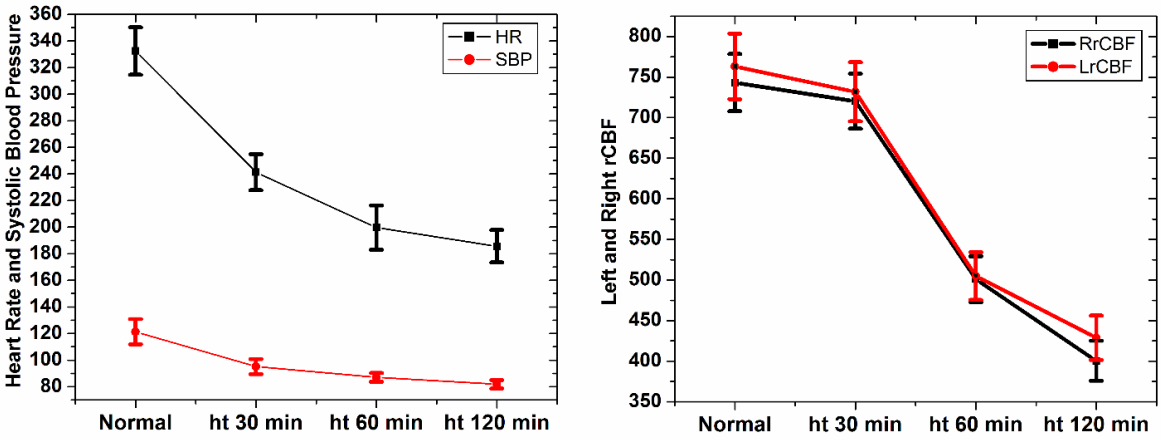

Fig. 7.1: Heart rate, SBP, and rCBF of normal and hypotension induced rat. (ht: hypotension induction by amlodipine and metoprolol administration; HR: heart rate; SBP: systolic blood pressure; rCBF: regional cerebral blood flow).

7.3.2 Cerebral infarction area

The TTC stained image and cerebral infarction area of control, positive control, BCCAO, and htBCCAO groups are illustrated in Fig 7.2. No infarction was observed in control and positive control groups. The 1/1 h I/R injury produced marked infarcts in both cortical and subcortical regions of rat forebrain as evidenced in TTC stained coronal brain sections of BCCAO and htBCCAO groups. The treatment with amlodipine and metoprolol induced noticeable infarction in rat brain of the htBCCAO group compared to BCCAO group ($p<0.001$).

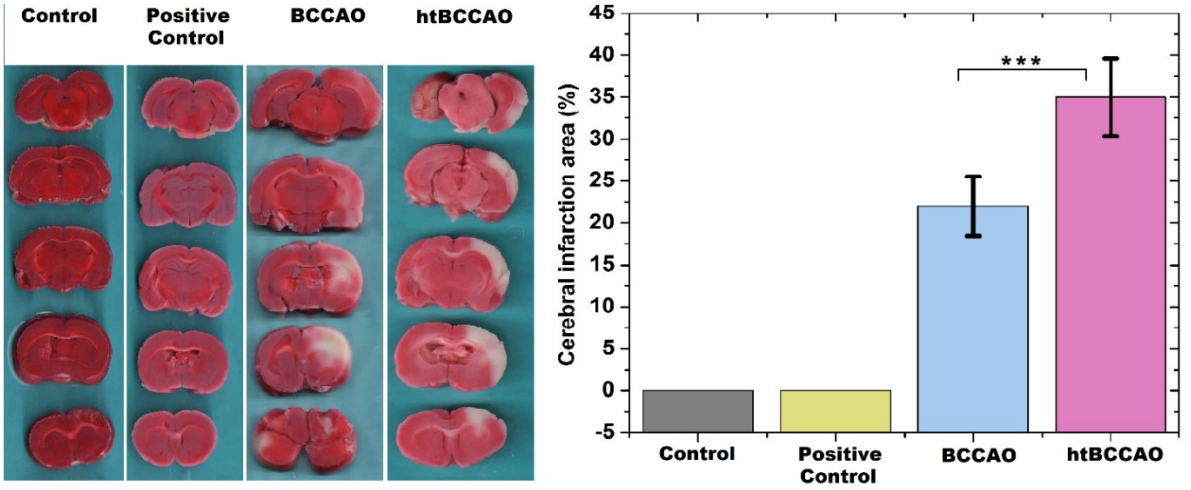

Fig 7.2. Visualization and quantification of cerebral infarction using TTC staining of rat's brain

7.3.3 Evans blue (EB) extravasation

The Evans blue dye assay was performed to check the integrity of BBB. EB Leakage was markedly increased in the injured brain regions of the htBCCAO group (2.2-fold) compared to BCCAO group ($p<0.001$). No leakage was observed in the control and positive control group. Fig. 7.3 represent the whole brains and their sections showing Evans blue extravasation in different groups.

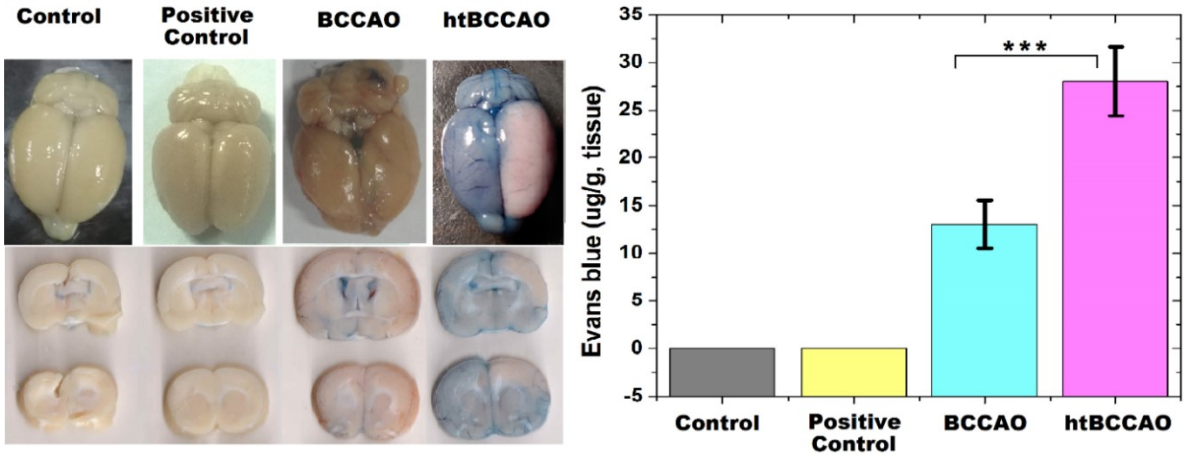

Fig. 7.3 Visualization and quantification of Evans blue (EB) extravasation in rat's brain.

7.3.4 Histological investigation

Hematoxylin and eosin (H&E) stains were used to evaluate the morphological and cellular changes in rat brain's cortex of all groups. H&E staining demonstrated that the infarction area characterized by the presence of vacuolation, small nuclei and deformed with cracked cytoblasts in the BCCAO and htBCCAO groups. Pre-administration of amlodipine and metoprolol (htBCCAO) followed by BCCAO increased the vacuolation, shrunk nuclei and deformed with cracked cytoblasts (Fig.7.4).

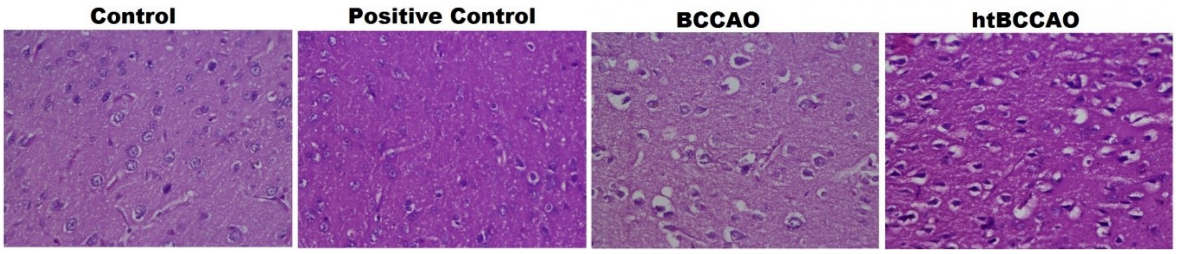

Fig. 7.4 Morphological and cellular changes in cortex visualized using H&E staining.

7.3.5 Apoptosis and necrosis detection

In apoptosis, the membrane phospholipid phosphatidylserine (PS) is accumulated and rapidly translocated from the cytoplasmic interface to the extracellular surface resulting loss of membrane asymmetry. This phenomena can be detected by Annexin V which is a calcium

dependent phospholipid binding protein and preferentially binds to negatively charged phospholipids including PS. In later stage of apoptosis and necrosis, the integrity of the plasma membrane is lost hence PI can penetrate and label the cells with a yellow-red fluorescence. The annexin V FITC and PI staining of brain cells of rats revealed that the apoptosis and necrosis occurs in all group's samples (Fig. 7.5). The control and positive control groups have less number of apoptotic and necrotic cells compared to BCCAO and htBCCAO groups. However, the htBCCAO group has the higher number of apoptotic and necrotic cells compared to BCCAO group.

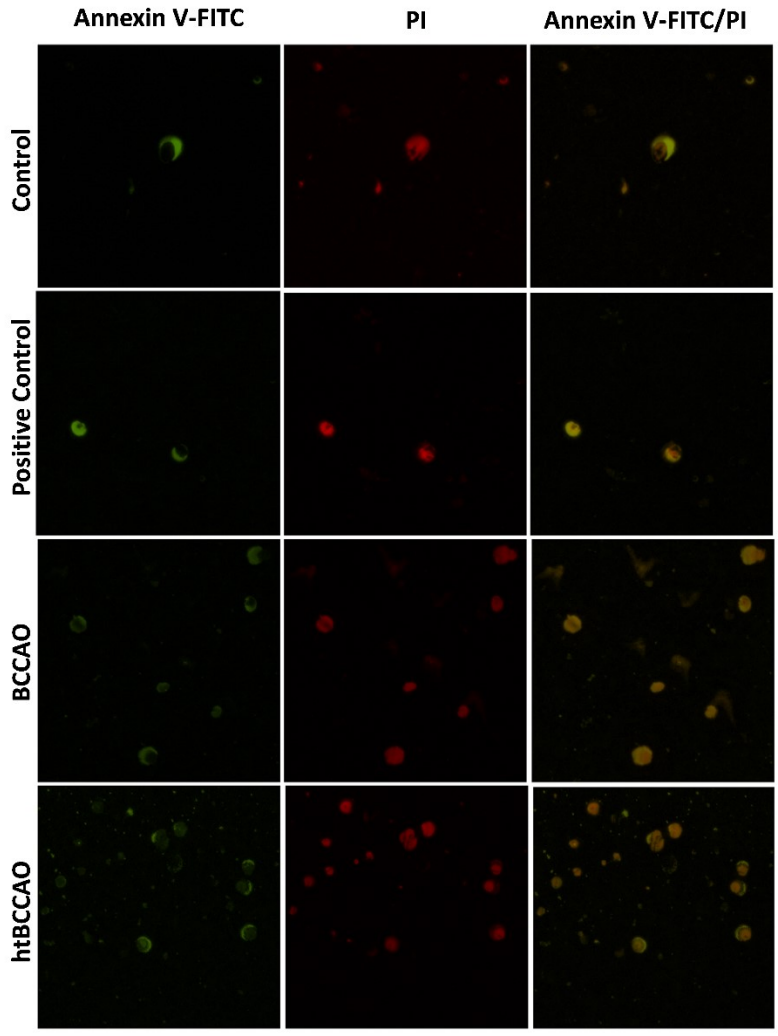

Fig. 7.5 Fluorescent microscopic image of Annexin VFITC/PI stained apoptotic brain cells

7.4 DISCUSSION

It is well established that BCCAO combined with systemic hypotension sufficient to induce reversible forebrain ischemia [36][39][98]. In this model, the hypotension has been induced artificially by maintaining the mean arterial blood pressure (MABP) near to 50mmHg using a surgical procedure that requires blood withdrawal after insertion of catheter tubing into the femoral artery or jugular vein. Blood is withdrawn immediately followed by BCCAO and is returned to the respective vessels after ending of the ischemic period. This model produces delayed neuronal injury in broad areas of the forebrain. In the present study, hypotension was induced in the rat through oral administration of Revelol AM that contain amlodipine and metoprolol (generally used for the treatment of HTN/BP). The administration of Revelol AM-induced hypotension and markedly reduced the BP and rCBF of animals (Fig. 7.1). The hypotension induced rats were subjected to BCCAO for 1h followed by 1h of reperfusion. Surprisingly, we found noticeable cerebral infarction and disruption of BBB in all animals of htBCCAO after 1/1h of I/R. Current study partially supports findings of a recently published report suggesting that acute BCCAO alone could not induce cerebral ischemia. Our results demonstrated that 1h of BCCAO followed by 1h of reperfusion could induce ischemia in the rat which is evident by marked lesions and BBB disruption in the forebrain of rats. However, induction of hypotension followed by 1/1h of I/R was sufficient to produce marked brain lesions and neuronal damage. Results suggested that hypotension coupled with BCCAO increased BBB permeability as well as produced larger infarction volume compared to BCCAO alone. Also, a comparatively high number of vacuolation and shrunk nuclei were observed in the infarcted cortical region of the htBCCAO group compared to BCCAO group. The apoptosis and necrosis study revealed that infarct area of htBCCAO group has more

number of annexin V and PI positive cells as compared to BCCAO group. The outcome of the present study is similar and supportive of previous studies [39][98] of 2VO/hypotension. However, this model requires multiple surgeries for blood withdrawing via cannulation followed by 2VO that make it more complicated. Whereas, present ischemic model omits the need for blood withdrawal from blood vessels and expertizes in cannulation. We also found least morbidity and good reproducibility of the neurological damage in this model. Moreover, several studies have been carried out with 2VO/BCCAO alone to demonstrate the neurological damage and subsequent neuroprotection, but no known research has been shown cerebral infarction and BBB damage except Iwasaki et al. [92]. We found that prolonged 2VO (at least 1h) followed by reperfusion can induce a varying degree of cerebral ischemia in rat with more than 70% of reproducibility (data not shown). As well as, hypotension coupled with BCCAO exaggerate the neuronal damage including apoptosis and necrosis.

7.5 CONCLUSION

The present study demonstrates that the administration of amlodipine and metoprolol can induce hypotension in rats. The hypotension coupled with bilateral common carotid artery occlusion for 1h followed by 1h of reperfusion is sufficient to produce cerebral infarction, BBB disruption, histopathological damage and brain cell death. This ischemic rat model is simple and has higher reproducibility as well as suitable for the molecular and biochemical studies and can be used for the evaluation of neuroprotective agents. Further, a study with more than 1h of reperfusion shall be conducted for analyzing the behavioral changes.