3.1 Abstract

Stroke is the primary cause of adult disability and sudden death. Ischemic stroke has been found to affect 80% of the population of a stroke patient. Recombinant tissue plasminogen activator (rtPA) is the widely used drug for the ischemic stroke with a narrow therapeutic window. Thus the development of new drug therapy is highly desirable for the treatment of cerebral ischemia. In the present study, we have prepared medicated barium and silver-containing bioactive glasses (BaBG & AgBG) and evaluated their potential against cerebral ischemic-reperfusion injury in rats. We found the antithrombotic activity and inhibitory effect of BaBG & AgBG on platelet aggregation in both in-vitro as well as ex-vivo. Further, both were effective in ameliorating MCAO induced neurobehavioral impairment, % infarction and cerebral blood flow. Middle cerebral artery occlusion (MCAO) model was used, followed by reperfusion after 2 hr of ischemia for the evaluation of the BaBG & AgBG against ischemic stroke. After reperfusion, BaBG (0.1, 0.5 and 1.0 mg/kg) and AgBG (1.0, 2.5 and 5.0 mg/kg) were given by iv route once daily for 28 days. Behavioral studies, including postural reflex, forelimb placing, and cylinder tests showed BaBG & AgBG attenuated the MCAO induced increase in average score and asymmetry score efficiently. Mean cerebral blood flow (CBF) was improved by treatment with BaBG (1 mg/kg) by 40 % & with AgBG (5 mg/kg) by 38 % of baseline at 4 h. Both BaBG & AgBG inhibited ADP induced platelet aggregation and reduced ischemic volume significantly. Brain histopathology had also shown lesser vacuolated interspaces and neuronal loss on treatment with BaBG & AgBG. Both BaBG & AgBG improve behavioral scores, mean CBF, reduce histopathological changes, and increase vascular endothelium growth factor after focal

cerebral ischemia in rats. Therefore, preclinical evidence points to BaBG & AgBG as a potential candidate for use in cerebral ischemic stroke.

Keywords:

Barium bioglass; silver bioglass, neurobehavioral; middle cerebral artery occlusion and platelet aggregation

3.2 Introduction

Stroke ranked, first to cause disability, second to cause dementia and third to cause death throughout the world (Whiteford et al. 2013). According to the American heart association, strokes are of three types; ischemic, hemorrhagic and transient ischemic attack, out of which ischemic stroke alone account for more than 80 % of stroke. Thus, the development of new drug therapy is highly desirable for the treatment of cerebral ischemia. Stroke is ischemic, when there is a sudden interruption of blood supply in the different part of the brain due to blood clot that impairs the supply of oxygen, glucose and other important nutrient resulting in energy failure and activates toxic intracellular pathways followed by the irreversible loss of nerve cell in the brain and neurological dysfunction (Deb et al. 2010). Reduction in CBF below a critical threshold level and duration determine the severity of ischemia in the brain. The severity of the ischemic injury is equivalent to % volume infarction which is detected by TTC stain, succinate dehydrogenase of mitochondria inner membrane losses a proton and appears red while infarction area not stained and appears pale. A blood clot causes further damage in the blood vessels due to disturbance in blood flow at the site of platelet aggregation and thrombi formation due to adherence of endogenous platelet activators such as collagen, adenosine diphosphate (ADP), and thrombin at the injury site. Antiplatelet therapy improves recovery

after stroke by preventing new clots formation. Thrombi are the source of thromboembolic complications of arteriosclerosis, heart attacks, strokes and peripheral vascular disease. Many antiplatelet agents have been developed and evaluated for their effects in preventing thrombosis since the inhibition of platelet function can be a promising approach for the prevention of thrombosis. Therefore we checked the antiplatelet activity of BaBG & AgBG against ADP induced Platelet aggregation. MCAO model of focal cerebral ischemia is one of the most used models as it mimics human stroke condition.

Bioactive glasses possess anti-inflammatory and antimicrobial application (Kargozar et al. 2019; US 20040289205 A1). In ischemic condition brain exposure of Bioglass significantly increased due to the opening of the blood-brain barrier (Pillai et al. 2009). Thus, the availability of these anti-inflammatory agents in the brain reduces inflammation of neuron, which will have significant value in the treatment of ischemia (Liu et al. 2014). Further bio-resorbable glass fibres facilitate peripheral nerve regeneration (Bunting et al. 2005) and angiogenesis (Day et al. 2005). Thus, the availability of bioactive glasses in the brain facilitates the regeneration of damaged nerve and the formation of the new blood vessel. Further, both barium and silver particles have an innate antiplatelet property. Barium effectively prevents the formation of secondary thrombus by blocking platelet IRKs channels (Inwardly rectifying potassium channels) which regulate ADP-induced platelet aggregation (Alagem et al. 2001 and Shankar et al. 2006), both in vivo and in vitro, in a concentration-dependent manner. Silver accumulates within platelet granules and reduces interplatelet proximity (Shrivastava et al. 2009). Thus we have prepared a bioactive glass containing barium & silver and tested them for the antiplatelet effect and further confirmed their potential in the treatment of cerebral ischemia.

In view of the above facts, we investigated the pharmacological effect of BaBG & AgBG for the treatment of cerebral ischemic stroke in a rat model. Bioactive glasses (BaBG & AgBG) and clopidogrel were given once daily through iv route after 2 hr of ischemia or at the time of reperfusion and continued up to the 28 days. Ischemic stroke was induced by middle cerebral artery occlusion (MCAO) for 2 h followed by reperfusion. Neurological assessment was done by postural reflex, forelimb placing and cylinder tests. To assess the recovery from cerebral ischemia mean cortical blood flow, infarct volume and brain histology were performed. The probable mechanism of action was evaluated through ADP induced platelet aggregation ex vivo. Clopidogrel served as a positive control.

3.3 Hypothesis

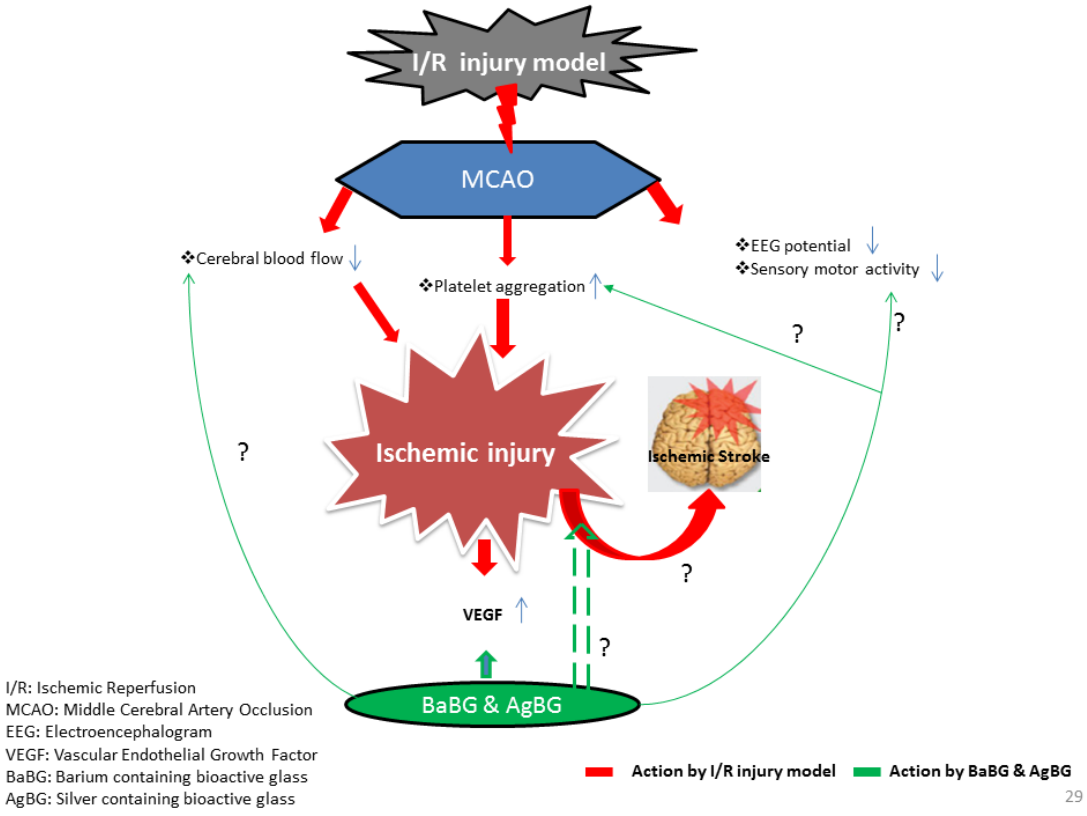

Figure 3.1 Proposed hypothesis: *Brain ischemic reperfusion injury was induced by middle cerebral artery occlusion. MCAO induces Ischemic stroke by inducing the platelet aggregation, reducing cerebral blood flow, EEG potential and sensory-motor activity of the rats. Since barium is reported to block IRKs channels (Inwardly rectifying potassium channels) which regulate ADP-induced platelet aggregation (Alagem et al. 2001, Shankar et al. 2006) and silver accumulates within platelet granules and reduces interplatelet proximity (Shrivastava et al. 2009)). Thus, we hypothesized that barium & silver-containing bioactive glass (BaBG & AgBG) inhibits platelet aggregation and treatment with BaBG & AgBG recovers from ischemic stroke by reducing cerebral blood flow, EEG potential and sensory-motor activity of the rats. Additionally, bioactive glass is reported to have VEGF stimulation property, and doping of metals like barium & silver in bioactive glasses significantly enhanced its VEGF stimulant property (Day et al. 2005). VEGF is associated with angiogenesis and vascular remodelling (Gandin et al. 2016). Increase in VEGF level has been reported to aid in the recovery of pathological as well as both neurological deficits in MCAO rodent model (Gandin et al. 2016). Therefore in the present study we have hypothesized barium & silver doped bioactive glass for the treatment of cerebral stroke and evaluated them against MCAO rodent model.*

3.4 Materials and Methods

3.4.1 Animals

Male Wistar rats (250–300g) from the central animal house of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India were used. Rats were housed under standard laboratory conditions, like free access to food and water, the standardized number of animals per cage, timely exchange of bedding and constant light and dark cycle. Animals were acclimatized for two weeks before the start of the experiment. Approval of Institutional Animal

Ethics Committee (Ref No. Dean/2015/CAEC/1421) was granted before the beginning of the experiment. All the experiments were performed as per the guidelines of laboratory animal care (National Research Council US Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011).

3.4.2 Materials

Clopidogrel was procured from Ranbaxy Research Laboratories, Gurgaon, India; pentobarbitone (Sigma-Aldrich, St.Louis, MO, USA); ADP (Hi-Media); Silica, calcium carbonate, sodium carbonate, barium carbonate, silver nitrate and ammonium dihydrogen orthophosphate were purchased from Loba Chemie, Mumbai, India.

3.4.3 Experimental design

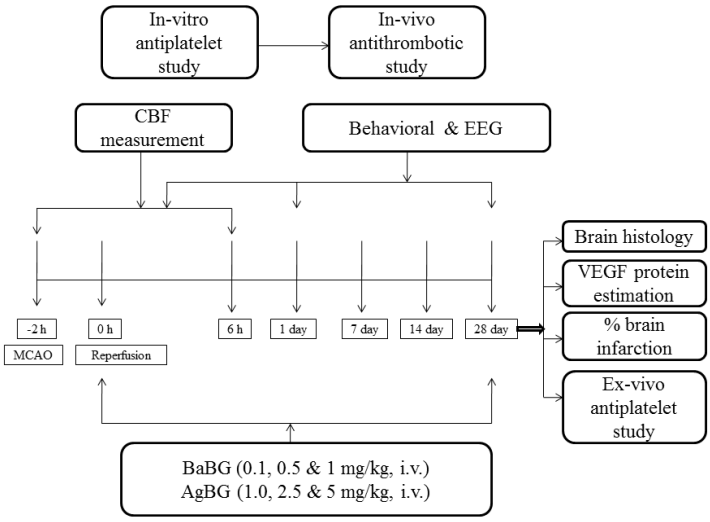

Figure 3.2 Schematic representation of the experimental design where i.v.: intravenous, h: hour, MCAO: middle cerebral artery occlusion and mg/kg: milligram/kilogram, CBF: cerebral blood flow, EEG: electroencephalogram, VEGF: vascular endothelial growth factor.

3.4.4 Bioactive glass preparation and its characterization

3.4.4.1 Preparation of BaBG & AgBG

Silica, calcium carbonate, sodium carbonate, ammonium dihydrogen orthophosphate and barium carbonate as a source of SiO_2, CaO, Na_2O, P_2O_5 and BaO in mol % of 44.9, 26.9, 24.3, 2.6 and 1.3 respectively were used for the preparation of BaBG. Similarly, silica, calcium carbonate, sodium carbonate, ammonium dihydrogen orthophosphate and silver nitrate as a source of SiO_2, CaO, Na_2O, P_2O_5 and ($AgNO_3$) in mol % of 44.9, 26.9, 24.3, 2.6 and 1.3 respectively were used for the preparation of AgBG. 45S5 bioglass® was prepared using 45.0, 24.5, 24.5, 6.0 mol% of SiO_2, CaO, Na_2O, P_2O_5 respectively. All the chemicals were of AnalaR grade and were purchased from LobaChemie, Mumbai, India. All chemicals were weighed and mixed for 30 min followed by melting it in 100 ml Pt-2% Rh crucible at 1400 ± 5 °C for 2 h in order to get the required bioactive glass. Further, the sample was annealed at 500 °C for 1 h, followed by cooling at a rate of 10 °C per minute till it attained the room temperature (Arepalli et al. 2015). The prepared sample was made into a fine powder using mortar and pestle. Finally, the sample was pulverized in a planetary ball mill (VB ceramics, India) for 6 h to make a fine powder of the average particle size of the bioactive glass in the range of micron.

3.4.4.2 Structural analysis of bioactive glass samples

Fourier transform infrared (FTIR) spectrometer was used for the determination of the functional group of bioactive glass samples in the frequency range of 600-4000 cm^{-1} at room temperature. The spectra of the prepared samples were recorded after placing the powdered bioactive glass sample directly in the instrument (Arepalli et al. 2015 and Paliwal et al. 2018).

3.4.4.3 Preparation of BaBG & AgBG suspension

Powdered BaBG sample was weighed and transfer to the glass beaker containing physiological saline and stir well with a glass rod. The sample was kept undisturbed for 72 hr at 37±2°C. After 72hr, PEG-400 was added to the prepared sample, and the pH of the above sample was adjusted to 7.4. The final volume was made up by physiological saline to get the concentration of 0.1, 0.5 and 1.0 mg/ml.

Similarly, AgBG aqueous extract was prepared by soaking it with saline for 72 hours of extraction at 37±2°C. Further PEG-400 (30 % v, v in saline) was added to get strength of 1.0 mg/ml, 2.5 mg/ml and 5 mg/ml for the dose of 1.0, 2.5 and 5.0 mg/kg respectively. PEG-400 was added for the even distribution of the final product.

3.4.4.4 Particle Size and surface morphology analysis of BaBG & AgBG suspension

The particle size was determined by using dynamic light scattering (Delsa Nano C, Beckman coulter). Samples were measured at a fixed angle of 165º at 25ºC (Landgraf et al. 2017). Average particle size was then determined in triplicate. The surface morphology of BaBG micro-suspension (1 mg/ml) & AgBG micro-suspension (5 mg/ml) was observed by a scanning electron microscopy (SEM, EVO LS 10, Carl Zeiss, Germany).

3.4.4.5 In-vitro blood compatibility of BaBG & AgBG suspension

Hemolysis experiments were performed to verify the *in-vitro* blood compatibility of the BaBG & AgBG micro-suspension to evaluate their potential for intravenous bolus administration (Venturini et al. 2016). Whole blood was collected from rats in a glass tube containing heparin sodium as an anticoagulant. Five different volumes ranging from 20 to 360 µL of BaBG suspension (1 mg/ml) were placed in glass tubes containing 7 mL of normal saline, 1 ml of rat blood was added and incubated at 37 ºC for 1 h. Similarly, Five different

volumes ranging from 20 to 360 µL of AgBG suspension (5 mg/ml) were placed in glass tubes containing 7 mL of normal saline, 1 ml of rat blood was added and incubated at 37 ºC for 1 h. The positive control was prepared with 1 ml of blood and 7 mL of distilled water. The negative control was obtained with 1 ml of blood and 7 mL of normal saline. After incubation, the samples were centrifuged for 15 minutes at 10000 rpm. The absorbance of the supernatant was detected at 540 nm (UV-Vis 1302131 PC Spectrophotometer, Biotech, USA). The analysis was performed in triplicates of suspension batches, and the percentage of hemolysis was calculated using the following equation

%Hemolysis = (ABS of formulation - ABS of negative control / ABS of positive control-ABS of negative control) ×100

Where ABS represents absorbance.

3.4.5 Drug treatment

3.4.5.1 BaBG Treatment

BaBG study was designed into two sets. The first set belongs to thrombosis rat experiment and the second set of study belongs to MCAO rat experiment.

Antithrombotic experiment consist of five groups (n=6 rats) i.e. control, clopidogrel (15 mg/kg), BaBG (0.1mg/kg), BaBG (0.5mg/kg), BaBG (1.0 mg/kg). Tail thrombosis was induced in rats using carrageenan of 0.9 mg/kg, iv. Thrombosis in the tail of all the rats was measured in term of length of the ischemic region of the tail using laser speckle Doppler imager.

MCAO experiment consist of six group (n=12) i.e control, vehicle, BaBG (0.1 mg/kg), BaBG (0.5 mg/kg), BaBG (1.0 mg/kg) and clopidogrel (15 mg/kg). BaBG suspension of 0.1,

0.5 and 1 mg/ml concentration using PEG-400 (30 % v,v in saline) was prepared for the dose of 0.1, 0.5, and 1 mg/kg respectively. Clopidogrel (15 mg/kg) was dispersed in PEG-400 (30 % v,v in saline). PEG-400 (30 % v,v in saline) of the 1 ml/kg was administered to the control and vehicle group. BaBG, clopidogrel and vehicle were administered at the time of reperfusion by i.v. route once a day, 7 days a week, for 28 consecutive days. Rat behavior (n=12) and cerebral blood flow (n=6) were recorded at the predetermined time intervals. Further, for infarct volume (n=5), VEGF (n=4) and histology (n=3) studies the brain samples were collected from the rats.

3.4.5.2 AgBG Treatment

Similar to BaBG treatment study, AgBG study was designed into two sets. The first set belongs to thrombosis rat experiment and the second set of study belongs to MCAO rat experiment.

Antithrombotic experiment consist of five groups (n=6 rats) i.e control, clopidogrel (15 mg/kg), AgBG (1.0 mg/kg), AgBG (2.5 mg/kg), AgBG (5.0 mg/kg). Tail thrombosis was induced in rats using carrageenan of 0.9 mg/kg, iv. Thrombosis in the tail of all the rats was measured in term of length of the ischemic region of the tail using laser speckle Doppler imager.

MCAO experiment consist of six group (n=12) i.e control, vehicle, AgBG (1.0 mg/kg), AgBG (2.5 mg/kg), AgBG (5.0 mg/kg) and clopidogrel (15 mg/kg). AgBG suspension of 1.0, 2.5 and 5.0 mg/ml concentration using PEG-400 (30 % v,v in saline) was prepared for the dose of 1.0, 2.5 and 5.0 mg/kg respectively. Clopidogrel (15 mg/kg) was dispersed in PEG-400 (30 % v,v in saline). PEG-400 (30 % v,v in saline) of the 1 ml/kg was administered

to the control and vehicle group. AgBG, clopidogrel and vehicle were administered at the time of reperfusion by i.v. route once a day, 7 days a week, for 28 consecutive days. Rat behavior (n=12) and cerebral blood flow (n=6) were recorded at the predetermined time intervals. Further, for infarct volume (n=5), VEGF (n=4) and histology (n=3) studies the brain samples were collected from the rats.

3.4.6 Middle cerebral artery occlusion in the rat

Focal ischemic reperfusion injury was induced by using the modified intraluminal method (Longa et al. 1989 and Paliwal et al. 2018). Briefly, rats were anaesthetized by intraperitoneal administration of pentobarbitone sodium (40 mg/kg) and then a middle neck incision was made to isolate the left common carotid artery without disturbing the vagus nerve. Further, the external and internal carotid arteries were isolated, and a monofilament (3-0) was introduced into the external carotid artery, and 18-22 mm of the filament was preceded towards the internal carotid artery to block the middle cerebral artery origin. After 2 h of occlusion, the filament was removed to cause the reperfusion injury (Belayev et al. 1996). Cerebral blood flow was measured to confirm blood vessel occlusion using a laser speckle blood flow imaging system (Omegazone OZ-2 STD, Japan). Throughout the experiment, the body temperature of the rats was maintained to 37 ± 0.5 ºC by using a heating pad.

3.4.7 Platelet aggregation study

3.4.7.1 Preparation of platelets

The rat blood was collected using heparinized syringes (1000U) by cardiac puncture from healthy rats for *in-vitro* antiplatelet study. For ex-vivo antiplatelet study, the rat blood was withdrawn from the MCAO experiments rats after 1 hr of the last treatment on day 28. Platelet-rich plasma (PRP) was isolated from the collected blood after centrifugation at 170 g

for 10 min at 4 °C (Dhurat et al. 2014). This procedure was done three times to collect the PRP from residual blood. Then platelets were counted by using platelet counter. PPP was obtained from residual blood by centrifugation at 1550 g for 25 min at 4 °C (Paliwal et al. 2018).

3.4.7.2 Platelet aggregation study

The inhibition of platelet aggregation by the bioactive glass was determined (*in-vitro* and *ex-vivo* in rat PRP) with Aggregometer (Chrono-Log Co., Havertown, PA, USA). PRP was used to set baseline value, and maximal transmission was set by using PPP. The PRP was incubated with BaBG & AgBG at 37 °C for 2 min in the aggregometer with stirring at the speed of 1200 rpm for intro study. However, for *ex-vivo* study, the PRP was isolated from the rats of MCAO experiments on 28 day, 1 hr of last dose. Then, ADP (10 µM) was added to the PRP to initiate the aggregation. Differences in the light transmission were recorded for 5 min after stimulation (Born et al. 1979).

3.4.8 Kappa-carrageenan-induced rat tail thrombosis model

The *in vivo* antithrombotic activity of BaBG & AgBG suspension was assessed in the k-carrageenan-induced thrombosis rat model (Hagimori et al. 2009). To assess the *in vivo* antithrombotic activity of BaBG suspension, a total of 30 male rats were randomly subdivided into 5 groups (control, BaBG 0.1, BaBG 0.5, BaBG 1.0 and 15.0 mg/kg clopidogrel), each group containing 6 rats. Group 1 rats were injected PEG-400 (30 % v,v in saline) in the volume of 1 ml/kg. The animals in groups 2, 3 and 4 were treated with graded concentrations (0.1, 0.5, and 1.0 mg/kg respectively) of BaBG and group 5 with 15.0 mg/kg clopidogrel. After 30 minutes of intravenous doses of BaBG or clopidogrel, the tails were ligated, and k-carrageenan 0.9-mg/kg was administered intravenously. Then, after 15

minutes, the ligatures were removed. The length of the blocked tail was measured after 6 hr of reperfusion using Laser Doppler Flow Meter imager.

Similarly, AgBG suspension was assessed in the k-carrageenan-induced thrombosis rat model (Hagimori et al. 2009) by using 5 groups (control, AgBG 1.0, AgBG 2.5, AgBG 5.0 and 15.0 mg/kg clopidogrel), each group containing 6 rats. Group 1 rats were injected PEG-400 (30 % v,v in saline) in the volume of 1 ml/kg. The animals in groups 2, 3 and 4 were treated with graded concentrations (1.0, 2.5 and 5.0 mg/kg respectively) of AgBG and group 5 with 15.0 mg/kg clopidogrel. After 30 minutes of intravenous doses of AgBG or clopidogrel, the tails were ligated, and k-carrageenan 0.9-mg/kg was administered intravenously. Then, after 15 minutes, the ligatures were removed. The length of the blocked tail was measured after 6 hr of reperfusion using Laser Doppler Flow Meter imager.

3.4.9 Electroencephalogram and cerebral blood flow recording

Cortical electrical activity is widely used as an indicator in cerebral ischemic-reperfusion (Wang et al. 2012). The change of EEG was monitored by Model MP45, BIOPAC System, INC, USA. Changes in CBF were recorded by a laser speckle blood flow imaging system (omegazone OZ-2 STD). Skull of anaesthetized rats were exposed by a midline scalp incision (Guo et al. 2010) and placed on the black sponge sheet located under the arm stand. Arm stand holds the CCD camera, the lens (ZM10-18, MF12) and the laser unit (780 nm for measurement and 650 nm for positioning). Raw speckle images were recorded from the skull surface using LSI Software (LSI ver.3.3, Omegawave, Inc., Tokyo, Japan) and average cerebral blood flow was determined by further analysis of images by using LIA Software (LIA ver.3.3, Omegawave, Inc., Tokyo, Japan). The black sheet does not reflect the laser

light and the effect makes the blood flow image clear (Guo et al. 2010 and Paliwal et al. 2018).

3.4.10 Infarct area measurement and brain histology

To determine the infarct volume, the rat brains were isolated and immediately kept at -20ºC for 5 min. The frozen brains were sliced with a thickness of 2 mm and incubated for 20 min at 37ºC in 2% TTC solution. After incubation, the slices were fixed in 10 % formalin. The images of the brain slices were captured to calculate the size of infarction using ImageJ software. Total infarct area was obtained by adding all infarcted area of slices. The brain oedema impairs the actual infarction volume; thus it needs to be corrected as follows: corrected infarct area = measured infarct area multiplied by {1-[(ipsilateral hemisphere area-contralateral hemisphere area)/contralateral hemisphere]}. The infarct volume was determined by multiplying the total infarct areas with the brain section thickness (Berti et al. 2002 and Wu et al. 2013).

Further, the rat brains were studied for histological examination. The isolated brains were fixed in 10% formaldehyde solution followed by paraffin embedding. The 5 µm sections were made and stained with hematoxylin and eosin (HE) and analyzed by light microscopy (Sarshoori et al. 2014). Cells (vacuole) in Histological slides were quantified with the help of ImageJ software (version 1.52), downloaded from National Institute of Health (NIH), USA (http//image.nih.go/ij). Cells on the margins of the image were not included for quantification.

3.4.11 Behavioral parameter

Standardized battery tests (Belayev et al. 1996) was used to quantify sensorimotor neurological function in all rats after 1 hr and 1, 14 and 28th day of treatment. Two separate tests were done. The postural reflex test was done to examine upper body posture while the animal was suspended by the tail (Bederson et al. 1986) and forelimb placing test was done to examine sensorimotor integration for visual, tactile and proprioceptive stimuli (De et al. 1989). Further, the cylinder test was performed to evaluate locomotor asymmetry of forelimbs (Hua et al. 2002). A higher value represents more severe motor deficits and vice versa. Brain functional activity was graded on a scale of 0-12 (normal score = 0, maximal score = 12) as previously described (Belayev et al. 1996).

3.4.12 VEGF estimation

The rat brains were isolated on the last day of the experiment, and the ischemic tissue, including core and penumbra, were extracting out. The extracted tissues (150 mg/ml) were homogenized in DMEM. Further, the homogenate tissues were centrifuged at 10,000 g for 10 min at 4 °C for the estimation of VEGF level (Gandin et al. 2016).

3.4.13 Statistical analysis

All the data were presented as Mean ± SEM. Repeated measures of Two-way ANOVA followed by Bonferroni's Post-hoc test was performed for the data analysis of CBF, EEG and behavioural observations. Further, one-way ANOVA was used for antithrombotic, antiplatelet, % brain infarction and VEGF data analysis with Student Newmann-Keuls Post-hoc test. A value of $p<0.05$ was accepted as significant.

3.5 Results

3.5.1 Evaluation of Bioactive glasses micro-suspension

3.5.1.1 Evaluation of BaBG micro-suspension

3.5.1.1.1 Structural and particle size analysis of BaBG sample

Fourier transform infrared transmittance spectra recorded in the frequency range of 600-4000 cm^{-1} of the bioactive glass samples using FTIR spectrometer is shown in Fig. 3.3. The vibrational bands obtained similar to transmission mode spectra as specified by earlier workers (Kansal et al. 2011). The 45S5 revealed the sharp bands successively at about 616, 796, 1076, 1512, 2312 and 3735 cm^{-1} indicating various functional groups. The band at 796 cm^{-1} is attributed to Si-O-Si symmetric stretching of non-bridging oxygen atoms between SiO_4 tetrahedral. A peak at 1076 cm^{-1} corresponds to Si-O-Si asymmetric stretching. The peak at 1512 cm^{-1} corresponds to the stretching mode of C-O vibration of O_3 groups. The infrared frequencies and related functional groups were also reported by ElBatal (ElBatal et al. 2003) in their bioglass® ceramic systems. The FTIR spectral bands of BaBG sample have clearly shown the similar behavior like 45S5 with a small change in the band intensities. The BaBG did not show any major changes in the FTIR transmission.

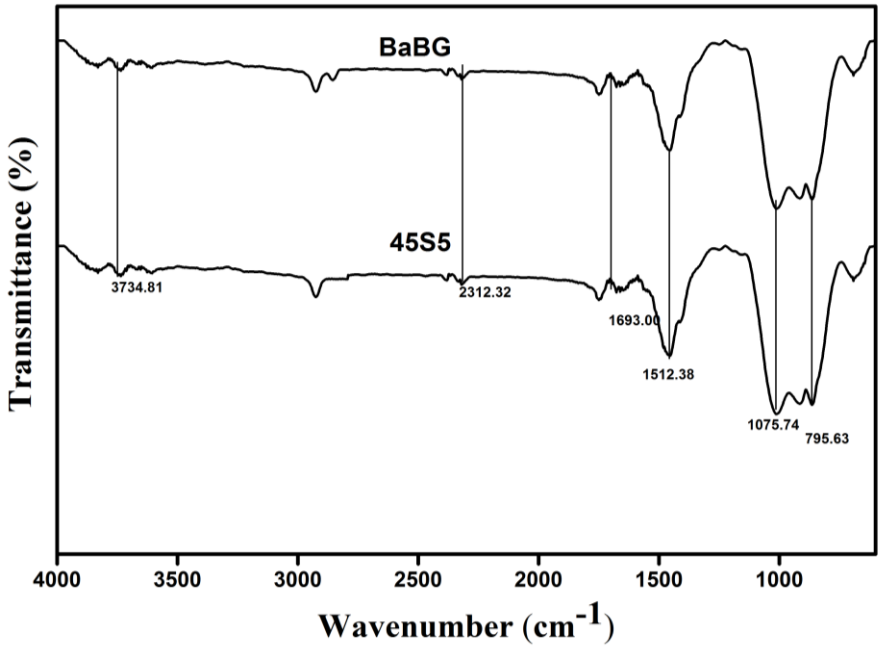

Figure 3.3 FTIR transmittance spectral analysis of BaBG and 45S5 samples

3.5.1.1.2 Particle size and surface morphology analysis of BaBG suspension

The particle size of the BaBG suspensions (0.1, 0.5 and 1.0 mg/ml) was determined using dynamic light scattering. It was found to be 1.388 ± 0.201, 1.988 ± 0.691 and 3.219 ± 0.640 micron respectively for 0.1, 0.5 and 1.0 mg/ml concentrations. The surface morphology of BaBG micro-suspension at highest dose i.e. 1 mg/ml is shown in Figure 3.4A as SEM images. As shown in Fig. 3.4A, the BaBG micro-suspension (1mg/ml) has micro particles of trapezoidal shape. The micrographs as given in Fig. 3.4B show the layer of polycrystalline particles formed on the surface of the microparticles. The developed crystals on the surface of the formulated microparticles are assumed to be hydroxy carbonate apatite (Arepalli et al. 2015).

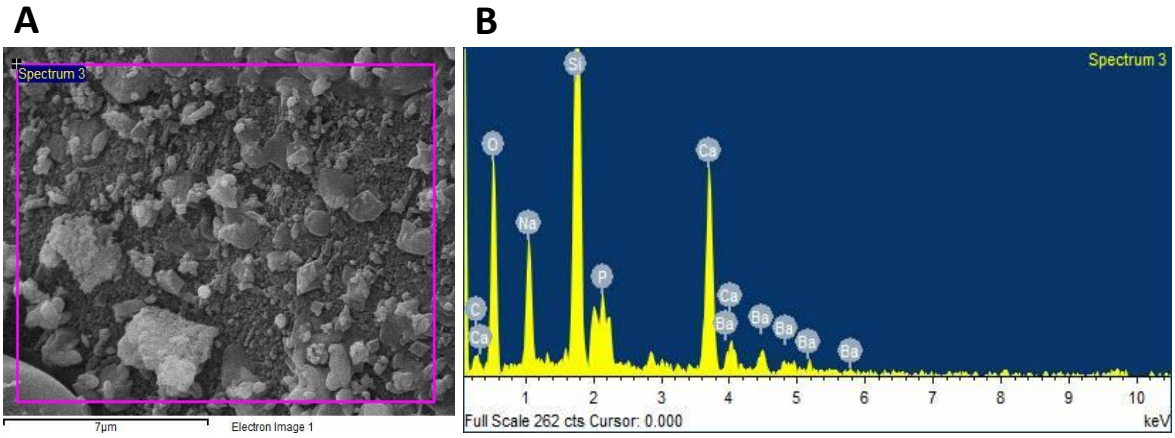

Figure 3.4 (A) SEM images and (B) Energy-dispersive X-ray spectroscopy of BaBG.

3.5.1.1.3 In-vitro blood compatibility of BaBG suspension

Hemolysis occurs due to the breakdown of red blood cells when it comes in contact with the components of the suspension; therefore, it is important to check the hemolysis potential of the suspension before the parenteral use. The percentage of hemolysis of blood due to BaBG micro-suspension was tested at the maximum dose i.e 1 mg/ml. The % hemolysis was found to be negligible. When rat blood incubated with 1 mg/ml of BaBG suspension of 20 µl, 40 µl, 80 µl, 160 µl and 320 µl for 1 hr its % hemolysis were 0.03 ± 0.01, 0.12 ± 0.02, 0.22 ± 0.03, 0.26 ± 0.03 and 0.41 ± 0.05% respectively.

3.5.1.2 Evaluation of AgBG micro-suspension

3.5.1.2.1 Structural and particle size analysis of AgBG sample

Fourier transform infrared transmittance spectra recorded in the frequency range of 600-4000 cm^{-1} of the bioactive glass samples using FTIR spectrometer is shown in Fig. 3.5 The vibrational bands obtained similar to transmission mode spectra as specified by earlier workers (Kansal et al. 2011). The 45S5 revealed the sharp bands successively at about 796,

1076, 1512, 2312 and 3735 cm^{-1} indicating various functional groups. A band at 796 cm^{-1} is attributed to Si-O-Si symmetric stretching of non-bridging oxygen atoms between SiO_4 tetrahedral. A peak at 1076 cm^{-1} corresponds to Si-O-Si asymmetric stretching. A peak at 1512 cm^{-1} corresponds to the stretching mode of C-O vibration of O_3 groups. The infrared frequencies and related functional groups were also reported by ElBatal (ElBatal et al. 2003) in their bioglass® ceramic systems. The FTIR spectral bands of AgBG sample have clearly shown the similar behavior like 45S5 with a small change in the band intensities. The AgBG did not show any major changes in the FTIR transmission.

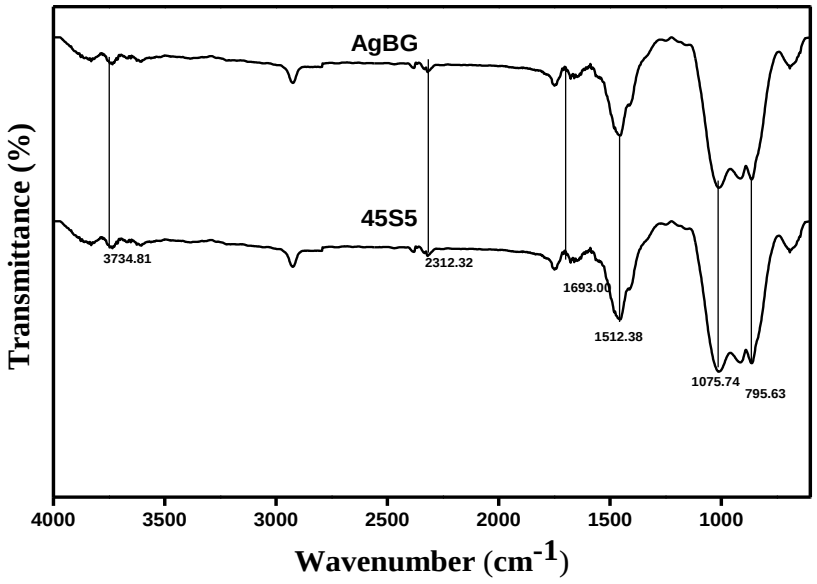

Figure 3.5 FTIR transmittance spectral analysis of AgBG and 45S5 samples

3.5.1.2.2 Particle size and surface morphology analysis of AgBG suspension

The particle size of the AgBG suspensions (1.0, 2.5 and 5.0 mg/ml) was determined using dynamic light scattering. It was found to be 1.316 ± 0.186, 1.754 ± 0.256 and 2.145 ± 0.340 micron respectively for 1.0, 2.5 and 5.0 mg/ml concentrations. The surface morphology of AgBG micro-suspension at highest dose i.e 5 mg/ml is shown in Figure 3.6A as SEM

images. As shown in Fig. 3.6A, the AgBG micro-suspension (5mg/ml) has micro particles of trapezoidal shape. The micrographs as given in Fig. 3.6B show the layer of polycrystalline particles formed on the surface of the microparticles. The developed crystals on the surface of the formulated microparticle are assumed to be hydroxy carbonate apatite (Arepalli et al. 2015).

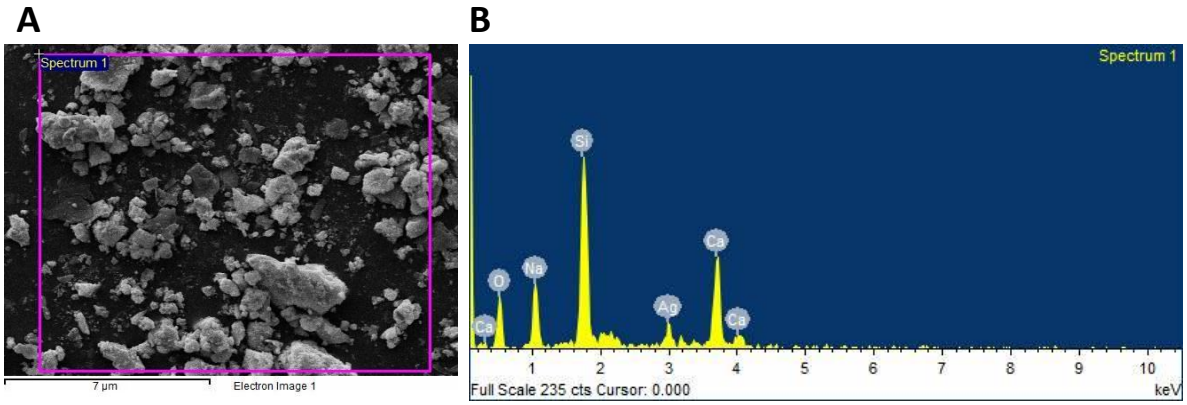

Figure 3.6 (A) SEM images and (B) Energy-dispersive X-ray spectroscopy of AgBG.

3.5.1.2.3 In-vitro blood compatibility of AgBG suspension

Hemolysis occurs due to the breakdown of red blood cells when it comes in contact with the components of the suspension; therefore, it is important to check the hemolysis potential of the suspension before the parenteral use. The percentage of hemolysis of blood due to AgBG micro-suspension was tested at the maximum dose i.e 5 mg/ml. The % hemolysis was found to be negligible. When rat blood incubated with 5 mg/ml of AgBG suspension of 20 µl, 40 µl, 80 µl, 160 µl and 320 µl for 1 hr its % hemolysis were 0.05 ± 0.01, 0.14 ± 0.03, 0.31 ± 0.04, 0.41 ± 0.06 and 0.56 ± 0.08% respectively.

3.5.2 Effect of Bioactive glasses on platelet aggregation

3.5.2.1 Effect of BaBG on platelet aggregation

Figure 3.7 illustrates the effect of BaBG on ADP-induced platelet aggregation. BaBG inhibited the platelet aggregation induced by ADP with the EC_{50} value of 7.81 µg/mL. The total blood volume in the rat is reported to 64 mL/kg (Sukbuntherng et al. 1996) and based on that the expected middle dose is 7.81 *64.00 = 499.84 µg/kg ≈ 0.5 mg/kg. Fig. 3.8 illustrates the effect of BaBG intravenous treatment (0.1, 0.5, and 1.0 mg/kg) on ADP-induced platelet aggregation in rat PRP. Treatment with BaBG at all three doses significantly inhibited ADP induced platelet aggregation in rat PRP compared to the vehicle [F (4, 25) = 94.38: P<0.05].

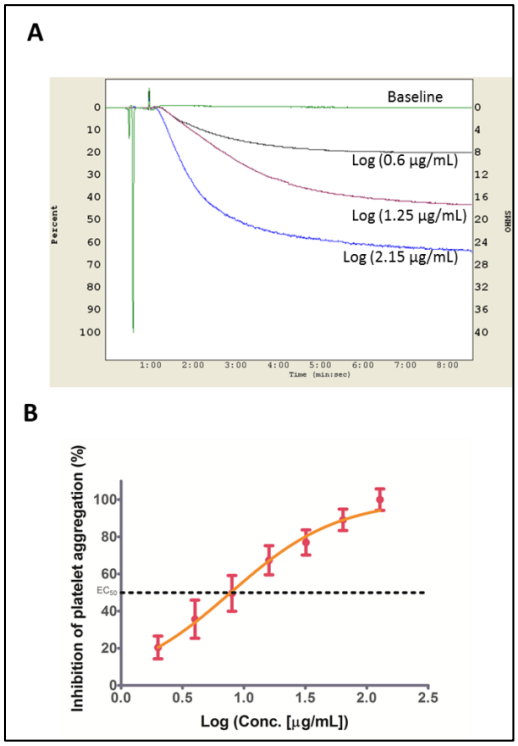

Figure 3.7: (A) shows representative aggregation curve, **(**B) *In-vitro* dose-response inhibition curves of BaBG on ADP-induced platelet aggregation in healthy rat PRP. *EC_{50} was defined as the concentration of the drug that inhibits platelet aggregation to 50 %. All values are mean ± SEM (n=6).*

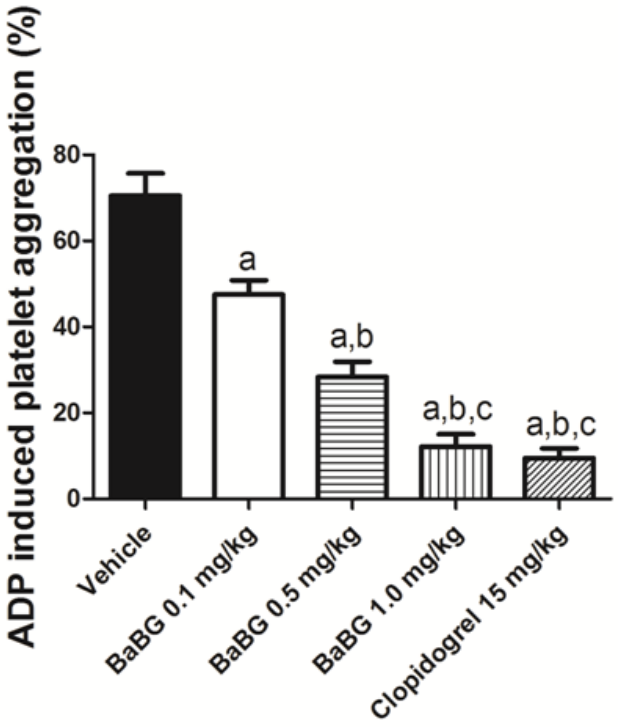

Figure 3.8 Ex-vivo effects of BaBG on ADP induced platelet aggregation in ischemic rat PRP. [a]P<0.05 compared to vehicle group, [b]P<0.05 compared to BaBG (0.1 mg/kg), and [c]P<0.05 compared to BaBG (0.5 mg/kg). All values are mean ± SEM (N=6) [One-way ANOVA followed by Student Newman-Keuls post-hoc test].

3.5.2.2 Effect of AgBG on platelet aggregation

Figure 3.9 illustrates the effect of AgBG on ADP-induced platelet aggregation. AgBG inhibited the platelet aggregation induced by ADP with the EC_{50} value of 39.06 µg/mL. The total blood volume in rat is reported to 64 mL/kg (Sukbuntherng et al. 1996) and based on that the expected middle dose is 39.06 *64.00 = 2499.84 µg/kg ≈ 2.5 mg/kg. Fig. 3.10 illustrates the effect of AgBG intravenous treatment (1.0, 2.5 and 5.0 mg/kg) on ADP-induced platelet aggregation in rat PRP. Treatment with AgBG at all three doses significantly inhibited ADP induced platelet aggregation in rat PRP compared to the vehicle [F (4, 25) = 28.72: P<0.05].

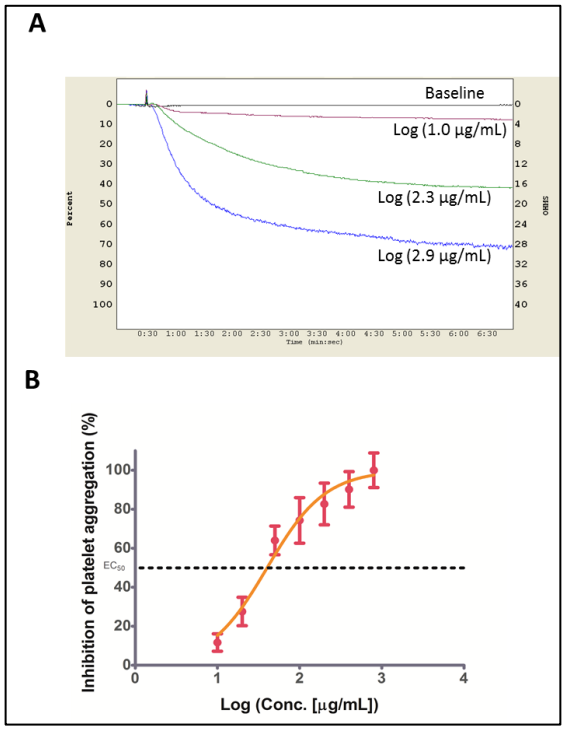

Figure 3.9: (A) shows representative aggregation curve, (B) *In-vitro* dose-response inhibition curves for AgBG inhibitory actions on ADP-induced platelet aggregation in healthy rat PRP. EC_{50} was defined as the concentration of the drug that inhibits platelet aggregation to 50 %. All values are mean ± SEM (n=6).

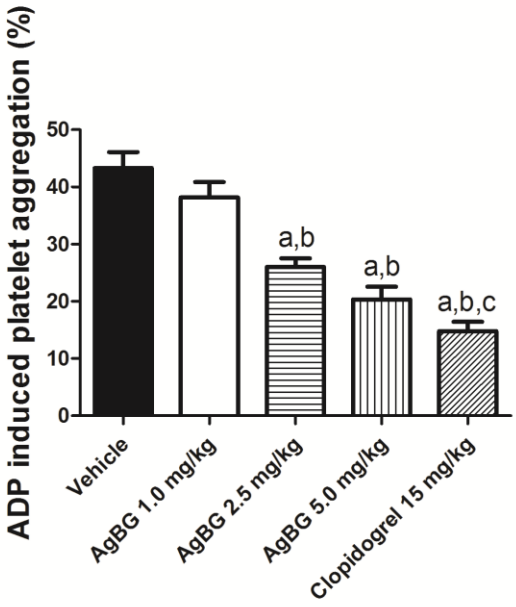

Figure 3.10 Ex-vivo effects of AgBG on ADP induced platelet aggregation in ischemic rat PRP. [a]P<0.05 compared to vehicle group, [b]P<0.05 compared to AgBG (1.0mg/kg), and [c]P<0.05 compared to AgBG (2.5 mg/kg). All values are mean ± SEM (N=6) [One-way ANOVA followed by Student Newman

3.5.3 Effect of Bioactive glasses on thrombosis

3.5.3.1 Effect of BaBG on thrombosis

BaBG (0.1, 0.5, and 1.0 mg/kg) significantly reduces the thrombus length generated by carrageenan in rat tail, as seen in figure 3.11. There was a significant change among groups [F (4, 24) = 196.8: P<0.05] as confirmed by the statistical analysis.

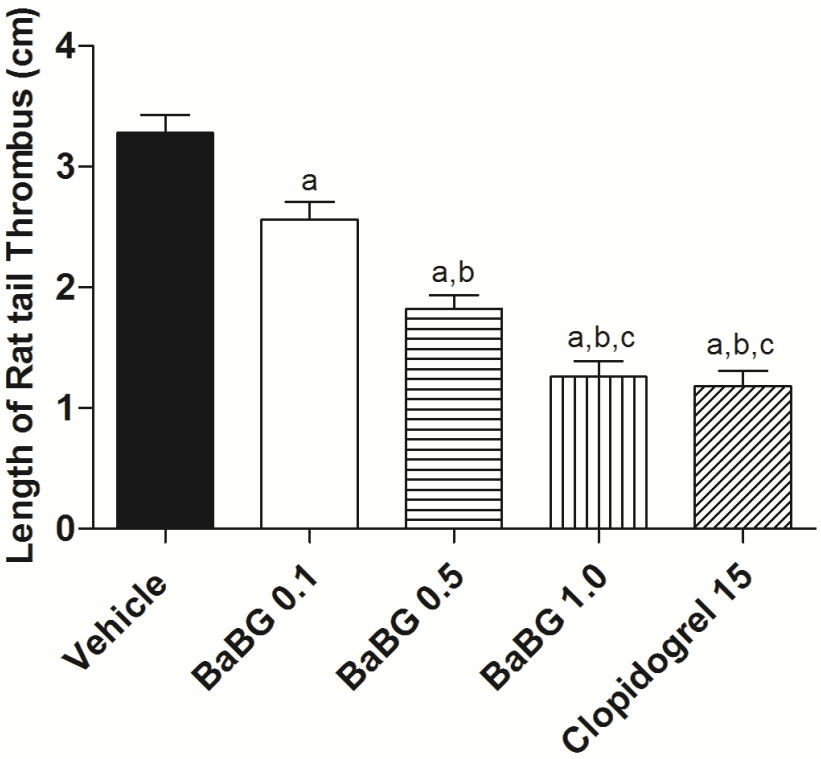

Figure 3.11 Bar represent the effect of BaBG on carrageenan induced tail thrombosis. All values are mean ± SEM (N=6). [a]P<0.05 compared to vehicle group, [b]P<0.05 compared to

BaBG 0.1, and $^{c}P<0.05$ compared to BaBG 0.5 [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

3.5.3.2 Effect of AgBG on thrombosis

AgBG (1.0, 2.5 and 5.0 mg/kg) significantly reduces the thrombus length generated by carrageenan in rat tail as seen in figure 3.12. There was a significant change among groups [$F(4, 24) = 86.54$: $P<0.05$] as confirmed by the statistical analysis.

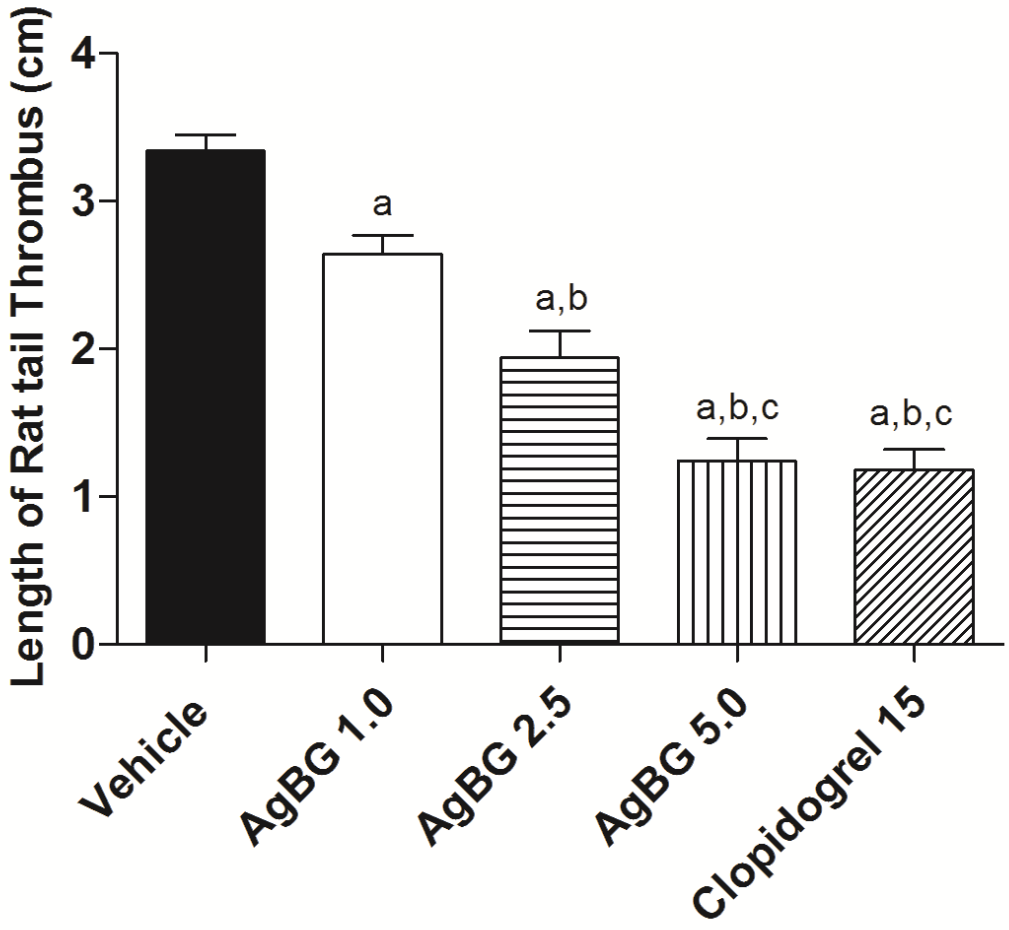

Figure 3.12 Bars represents the effect of AgBG on carrageenan-induced tail thrombosis. All values are mean ± SEM (N=6). aP<0.05 compared to the vehicle group, bP<0.05 compared

to AgBG 1.0, and cP<0.05 compared to AgBG 2.5 [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

3.5.4 Effect of Bioactive glasses on Mean cerebral blood flow

3.5.4.1 Effect of BaBG on Mean cerebral blood flow

Figure 3.13 illustrates the effect of BaBG (0.1, 0.5, and 1.0 mg/kg) on changes in mean CBF in MCAO rats. BaBG significantly recovered mean CBF after 4 h of reperfusion compared to vehicle groups [F (24, 175) = 12.02; P<0.05]. The increase in mean CBF from baseline at 6 h in rats treated with BaBG with a dose of 0.1, 0.5 and 1.0 mg/kg was 67%, 78 % and 83 % respectively.

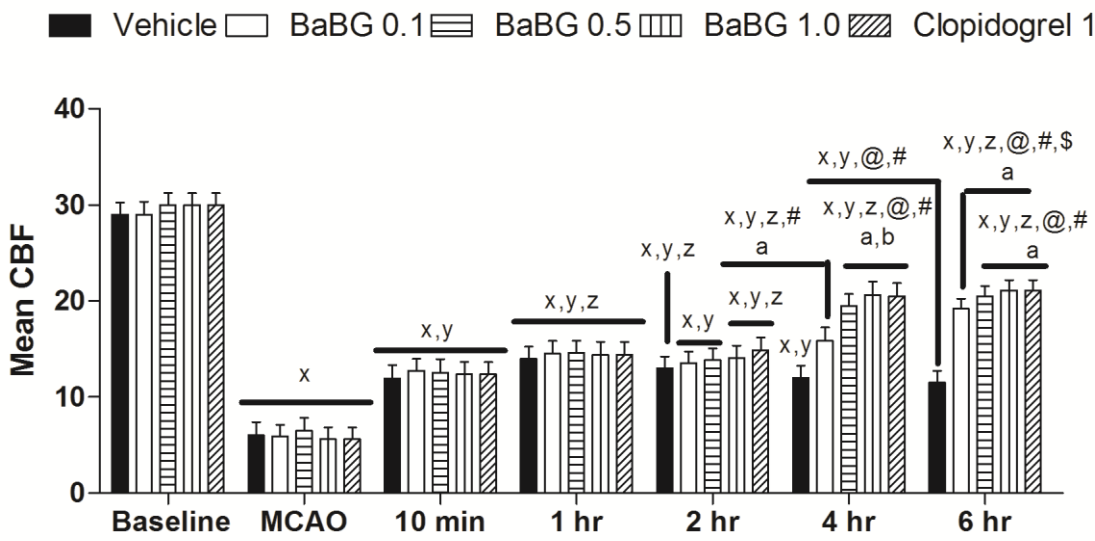

Figure 3.13 Bars represent the effect of BaBG on changes in mean CBF in MCAO rats. All values are mean ± SEM (N=06). [a]P<0.05 compared to vehicle, [b]P<0.05 compared to BaBG (0.1 mg/kg), [x]P < 0.05 compared to baseline, [y]P < 0.05 compared to MCAO, [z]P < 0.05 compared to 10 min, [@]P < 0.05 compared to 1 hr, [#]P < 0.05 compared to 2 hr and [$]P < 0.05 compared to 4 hr [Two-way ANOVA followed by Bonferroni Post-hoc test].

3.5.4.2 Effect of AgBG on Mean cerebral blood flow

Figure 3.14 illustrates the effect of AgBG (1.0, 2.5 and 5.0 mg/kg) on changes in mean CBF in MCAO rats. AgBG significantly recovered mean CBF after 4 h of reperfusion compared to vehicle groups [F (24, 175) = 10.54; P<0.05]. The increase in mean CBF from baseline at 6 h in rats treated with AgBG with a dose of 1.0, 2.5 and 5.0 mg/kg was 43%, 51 % and 64 % respectively.

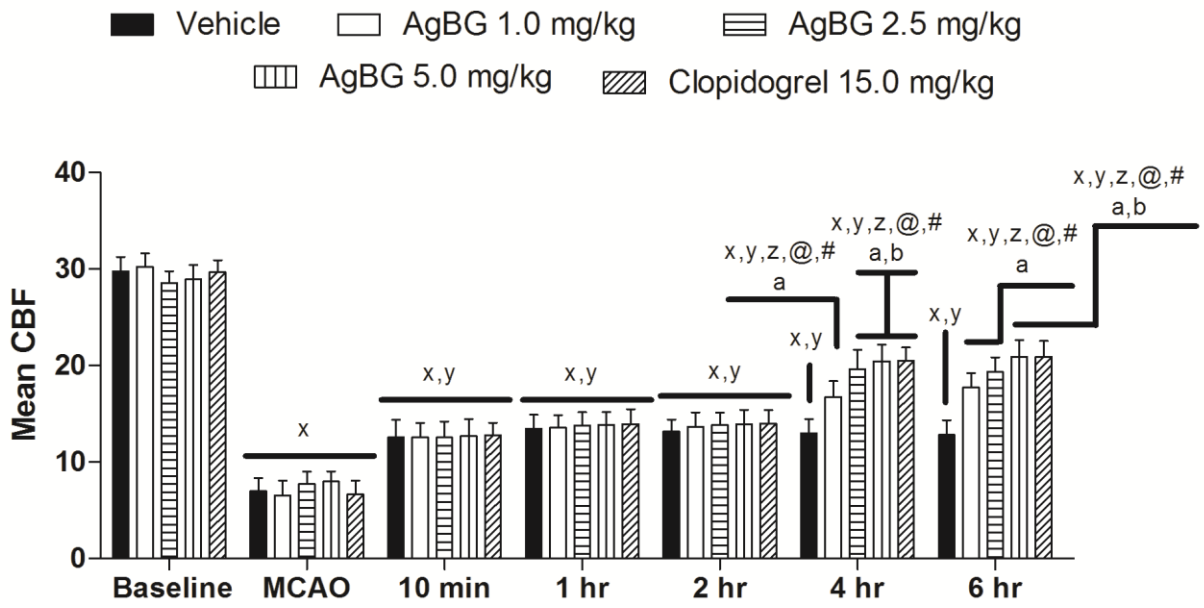

Figure 3.14 Bars represent the effect of AgBG on changes in mean CBF in MCAO rats. All values are mean ± SEM (N=06). aP<0.05 compared to vehicle, bP<0.05 compared to AgBG (1.0mg/kg), xP < 0.05 compared to baseline, yP < 0.05 compared to MCAO, zP < 0.05 compared to 10 min, @P < 0.05 compared to 1 hr, #P < 0.05 compared to 2 h and $P < 0.05 compared to 4 h [Two-way ANOVA followed by Bonferroni Post-hoc test].

3.5.5 Effect of Bioactive glasses on EEG recovery after middle cerebral artery occlusion.

3.5.5.1 Effect of BaBG on EEG recovery after middle cerebral artery occlusion.

Figure 3.15 illustrates the effect of BaBG suspension (0.1, 0.5, and1.0 mg/kg) in recovery of EEG in MCAO rats. MCAO significantly reduces the cortical electrical activity in the brain. Treatment with BaBG suspension at the dose at 1.0 mg/kg significantly recovered MCAO-induced EEG changes after 28 of reperfusion compared to vehicle groups [F (20, 120) = 5.069; P<0.05].

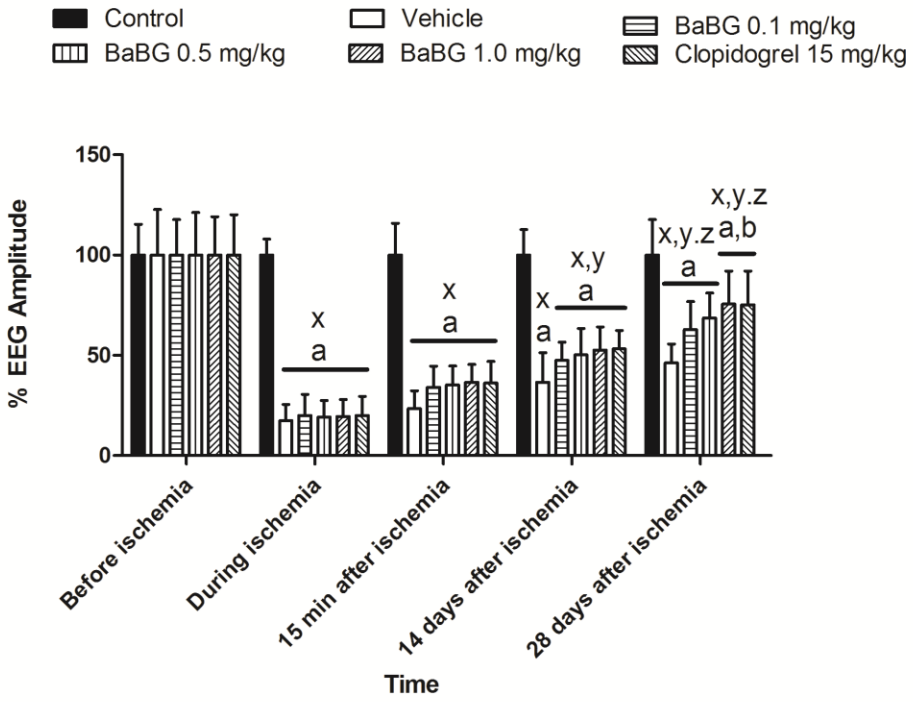

Figure 3.15 Bars represents the effect of BaBG on EEG changes in MCAO rats. All values are mean ± SEM (N=5). [a]P<0.05 compared to control group, [b]P<0.05 compared to vehicle, [x]P < 0.05 compared to before ischemia, [y]P < 0.05 compared to during ischemia and [z]P < 0.05 compared to 15 min after ischemia [Two-way ANOVA followed by Bonferroni post-hoc test].

3.5.5.2 Effect of AgBG on EEG recovery after middle cerebral artery occlusion

Figure 3.16 illustrates the effect of AgBG suspension (1.0, 2.5, and 5.0 mg/kg) in the recovery of EEG in MCAO rats. MCAO significantly reduces the cortical electrical activity in the brain. Treatment with AgBG suspension at the dose at 5.0 mg/kg significantly recovered MCAO-induced EEG changes after 28 of reperfusion compared to vehicle groups [F (20, 120) = 7.140; P<0.05].

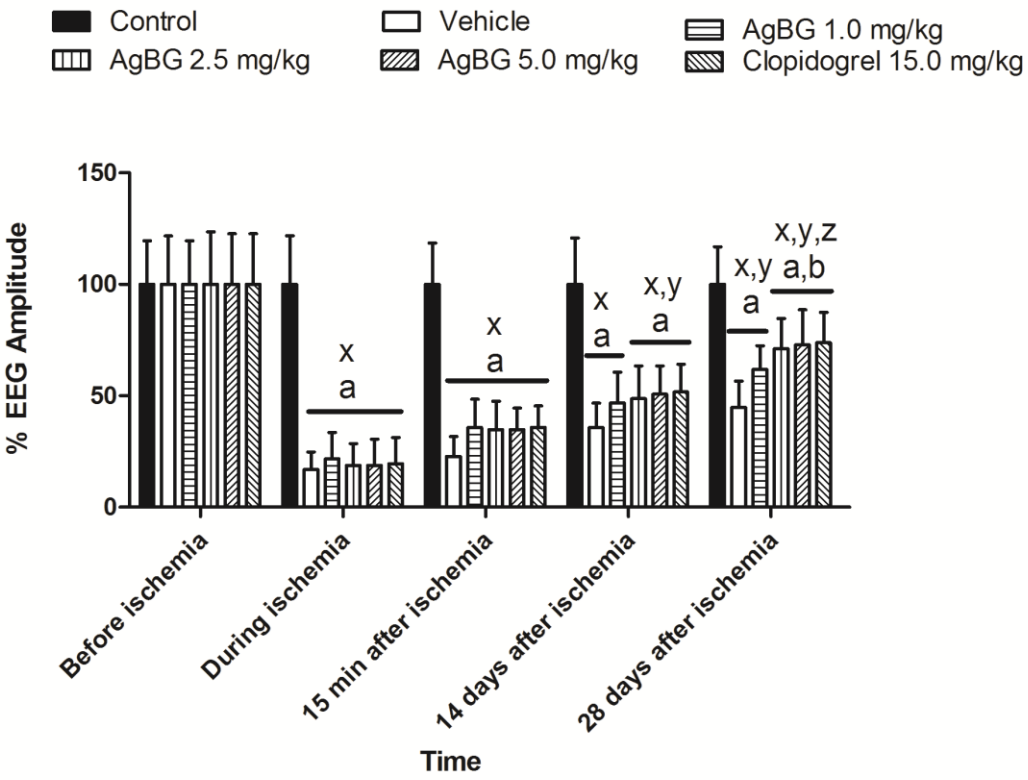

Figure 3.16 Bars represents the effect of AgBG on EEG changes in MCAO rats. All values are mean ± SEM (N=5). aP<0.05 compared to control group, bP<0.05 compared to vehicle, xP < 0.05 compared to before ischemia, yP < 0.05 compared to during ischemia and zP < 0.05 compared to 15 min after ischemia [Two-way ANOVA followed by Bonferroni post-hoc test].

3.5.6 Effect of Bioactive glasses on infarction volume after MCAO.

3.5.6.1 Effect of BaBG on infarction volume after MCAO.

Figure 3.17 illustrates the effect of BaBG suspension (0.1, 0.5, and 1.0 mg/kg) on infarct volumes in MCAO rats. One-way analysis of variance followed by Bonferroni post-hoc tests revealed a significantly smaller infarct volume in BaBG treated group in contrast to the vehicle [$F(5, 29) = 134.0$; $P<0.05$] as shown in fig 3.17 (B).

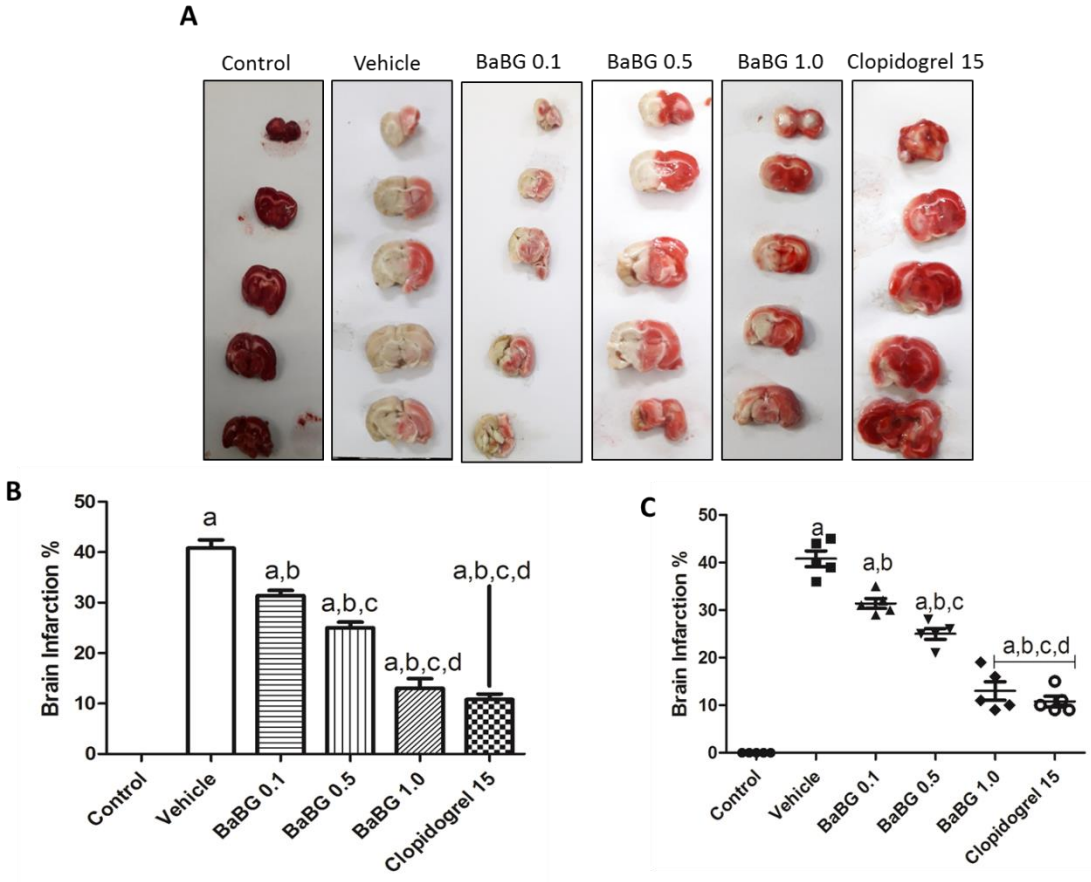

Figure 3.17 Effect of BaBG on brain infarct volume in MCAO rats. (**A)** Represents the stained images for groups, (**B**) and (**C)** represent the BARS and dot plot respectively. All values are mean ± SEM (N=5). [a]$P<0.05$ compared to control group, [b]$P<0.05$ compared to vehicle, [c]$P<0.05$ compared to BaBG 0.1 and [d]$P<0.05$ compared to BaBG 0.5 [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

3.5.6.2 Effect of AgBG on infarction volume after MCAO

Figure 3.18 illustrates the effect of AgBG suspension (1.0, 2.5, and 5.0 mg/kg) on infarct volumes in MCAO rats. One-way analysis of variance followed by Bonferroni posttests revealed a significantly smaller infarct volume in AgBG treated group in contrast to the vehicle [F (5, 29) = 118.0; P<0.05] as shown in fig 3.18(B).

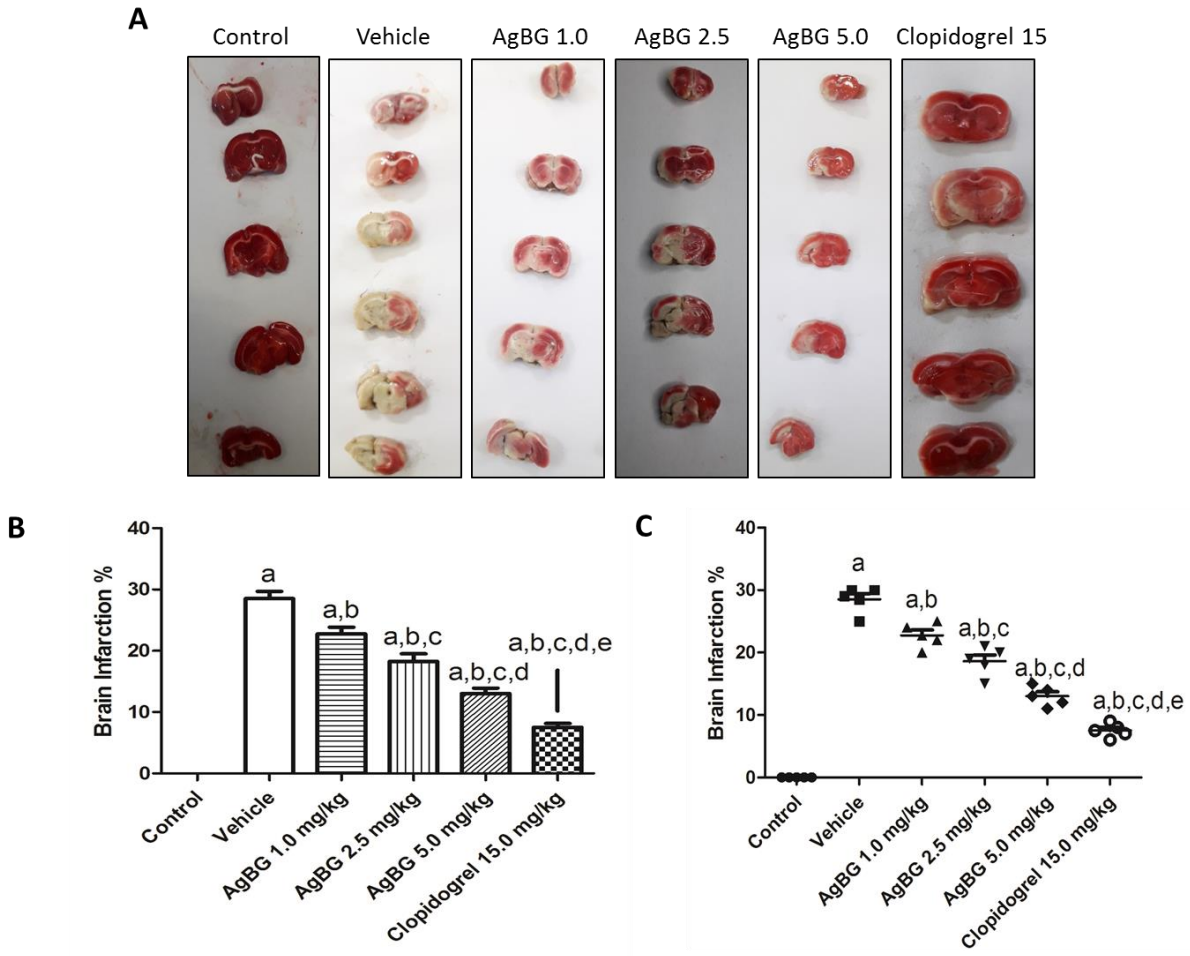

Figure 3.18 Effect of AgBG on brain infarct volume in MCAO rats. (A) Represents the stained images for groups, (**B)** and (**C)** represent the BARS and dot plot, respectively. All values are mean ± SEM (N=5). [a]P<0.05 compared to control group, [b]P<0.05 compared to vehicle, [c]P<0.05 compared to AgBG 1.0 and [d]P<0.05 compared to AgBG 2.5 [One-way ANOVA followed by Student Newmann-Keuls Post-hoc test].

3.5.7 Effect of Bioactive glasses on behavioral changes after MCAO.

3.5.7.1 Effect of BaBG on behavioral changes after MCAO.

3.5.7.1.1 Total Neurological Score

To determine the effect of BaBG on motor performance in MCAO rats, postural reflex test and limb placing test were performed. Figure 3.19. A shows the effect of BaBG (0.1, 0.5 and 1.0 mg/kg) and clopidogrel (15 mg/kg) on the total neurological score obtained from postural reflex test and limb placing test on MCAO rats. Statistical analysis showed significant differences in a total score between groups [$F(4, 220) = 123.2$; $P<0.05$].

3.5.7.1.2 Cylinder Test

To determine the effect of BaBG on asymmetry in forelimb usage in MCAO rats, the cylinder test was performed. BaBG significantly reduces the asymmetry score in MCAO rats, and there was a significant difference among the groups [$F(4, 220) = 169.3$; $P<0.05$] as depicted in figure 3.19.B.

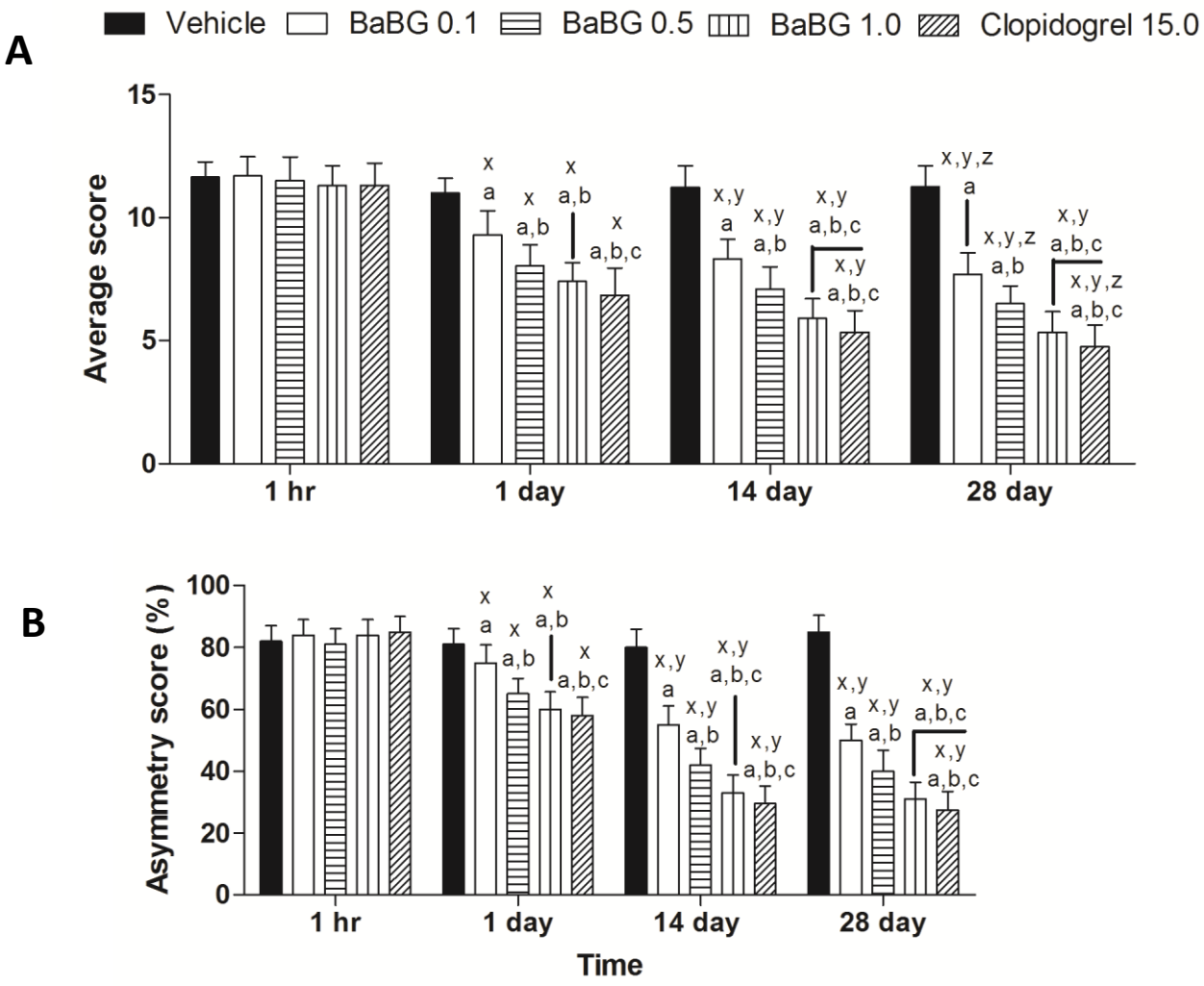

Figure 3.19 Bars represent the effect of BaBG on changes in behavioral test in MCAO rats. Panel (A) shows changes in the average neurological and (B) Asymmetry score after 2-h MCAO followed by 28 days reperfusion. All values are mean ± SEM (N=12). $^{a}P<0.05$ compared to vehicle group, $^{b}P<0.05$ compared to BaBG 0.1, $^{c}P<0.05$ compared to BaBG 0.5 , $^{x}P < 0.05$ compared to 1 h, $^{y}P < 0.05$ compared to 1 day and $^{z}P < 0.05$ compared to 14 day[Two-way ANOVA followed by Bonferroni Post-hoc test].

3.5.7.2 Effect of AgBG on behavioral changes after MCAO

3.5.7.2.1 Total Neurological Score

To determine the effect of AgBG on motor performance in MCAO rats, postural reflex test and limb placing test were performed. Figure 3.20. A shows the effect of AgBG (1.0, 2.5 and

5.0 mg/kg) and clopidogrel (15 mg/kg) on the total neurological score obtained from postural reflex test and limb placing test on MCAO rats. Statistical analysis showed significant differences in total score between groups [F (4, 220) = 131.1; P<0.05].

3.5.7.2.2 Cylinder Test

To determine the effect of AgBG on asymmetry in forelimb usage in MCAO rats, the cylinder test was performed. AgBG significantly reduces the asymmetry score in MCAO rats, and there was a significant difference among the groups [F (4, 220) = 156.4; P<0.05] as depicted in figure 3.20.B.

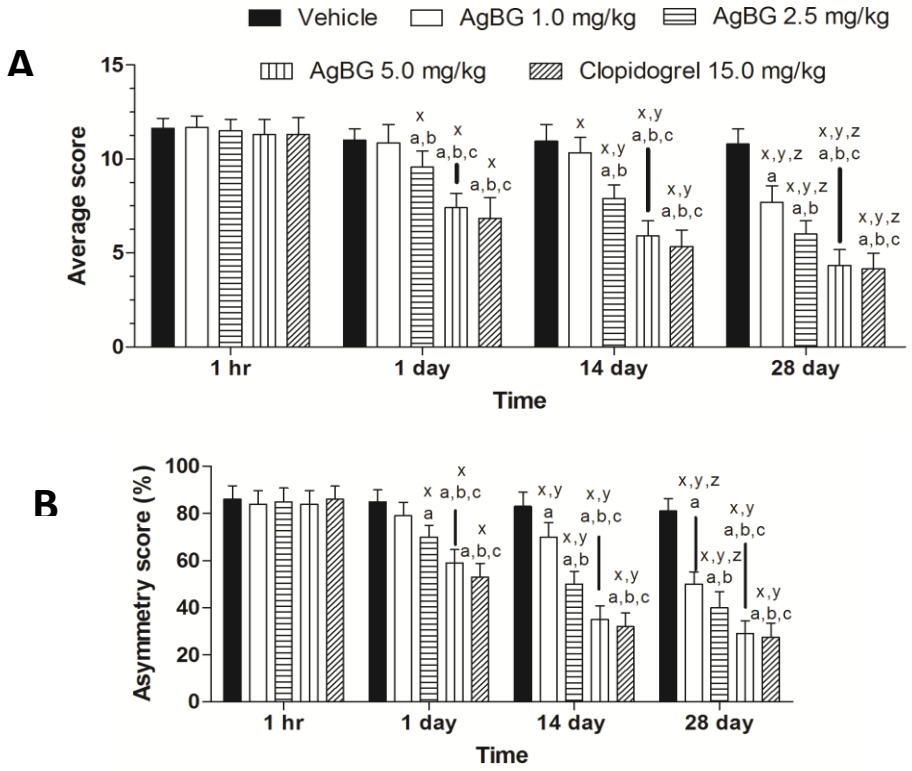

Figure 3.20 Bars represent the effect of AgBG on changes in behavioral test in MCAO rats. Panel (A) shows changes in the average neurological and (B) Asymmetry score after 2-h MCAO followed by 28 days reperfusion. All values are mean ± SEM (N=12). $^{a}P<0.05$ compared to vehicle group, $^{b}P<0.05$ compared to AgBG 1.0, $^{c}P<0.05$ compared to AgBG 2.5 , $^{x}P < 0.05$ compared to 1 h, $^{y}P < 0.05$ compared to 1 day and $^{z}P < 0.05$ compared to 14 day[Two-way ANOVA followed by Bonferroni Post-hoc test].

3.5.8 Histological examination

3.5.8.1 Histological examination of BaBG study

Figure 3.21 represented the results of histological examination of the cortex of the brain in different groups. Staining of ischemic brain tissues by H.E. revealed the absence of intact neurons, the presence of multiple vacuolated interspaces and neuronal loss. However, the corresponding areas of brain sections from the BaBG treatment group showed the presence of intact neurons in between the vacuolated spaces. Number of cells with vacuole in Figure 3.21 are followings A-control (9), B-vehicle(155), C-BaBG 0.1 mg/kg (79), D-BaBG 0.5 mg/kg(58), E-BaBG 1.0 mg/kg (29), and F-clopidogrel 15 mg/kg (19).Further, SEM-EDS image of the cortex of the ischemic brain at the dose of 1.0 mg/kg BaBG on the last day of the experiment shown the brain penetration of BaBG in ischemic rat brain as confirmed by Figure 3.22.

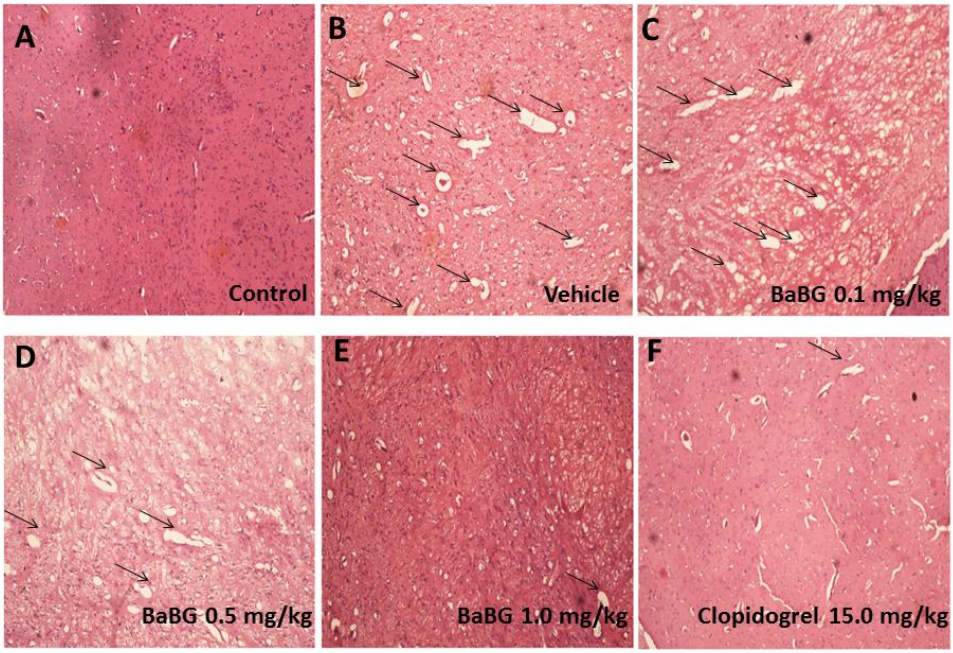

Figure 3.21 Effects of BaBG on cortex of the ischemic brain. (A-F) show the histology of different rat brain slices stained with (H.E., haematoxylin and eosin). A-control, B-vehicle, C-BaBG 0.1 mg/kg, D-BaBG 0.5 mg/kg, E-BaBG 1.0 mg/kg, and F-clopidogrel 15 mg/kg. Number of cells with vacuole in Figure 3.21 are followings A-control (9), B-vehicle (155), C-BaBG 0.1 mg/kg (79), D-BaBG 0.5 mg/kg (58), E-BaBG 1.0 mg/kg (29), and F-clopidogrel 15 mg/kg (19).The arrows indicate cavitation of the cytoplasm (n=3).

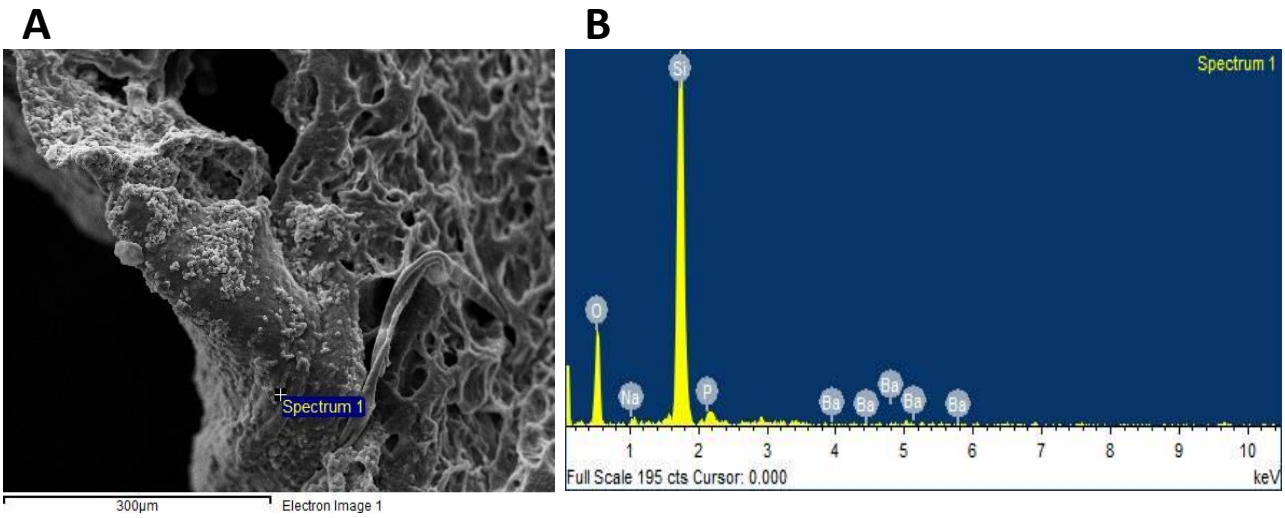

Figure 3.22 (A) SEM images and (B) Energy-dispersive X-ray spectroscopy of cortex of the ischemic brain at the dose of 1.0 mg/kg BaBG on the last day of the experiment.

3.5.8.1 Histological examination of AgBG study

Figure 3.23 represented the results of histological examination of the cortex of the brain in different groups. Staining of ischemic brain tissues by H.E. revealed the absence of intact neurons, the presence of multiple vacuolated interspaces and neuronal loss. However, the corresponding areas of brain sections from the AgBG treatment group showed the presence of intact neurons in between the vacuolated spaces. Number of cells with vacuole in Figure 3.23 are followings A-control (1), B-vehicle (34), C-AgBG 1.0 mg/kg (12), D-AgBG 2.5 mg/kg (6), E-AgBG 5.0 mg/kg (3), and F-clopidogrel 15 mg/kg (2).Further, SEM-EDS image of the cortex of the ischemic brain at the dose of 5.0 mg/kg AgBG on the last day of

the experiment shown the brain penetration of AgBG in ischemic rat brain as confirmed by Figure 3.24.

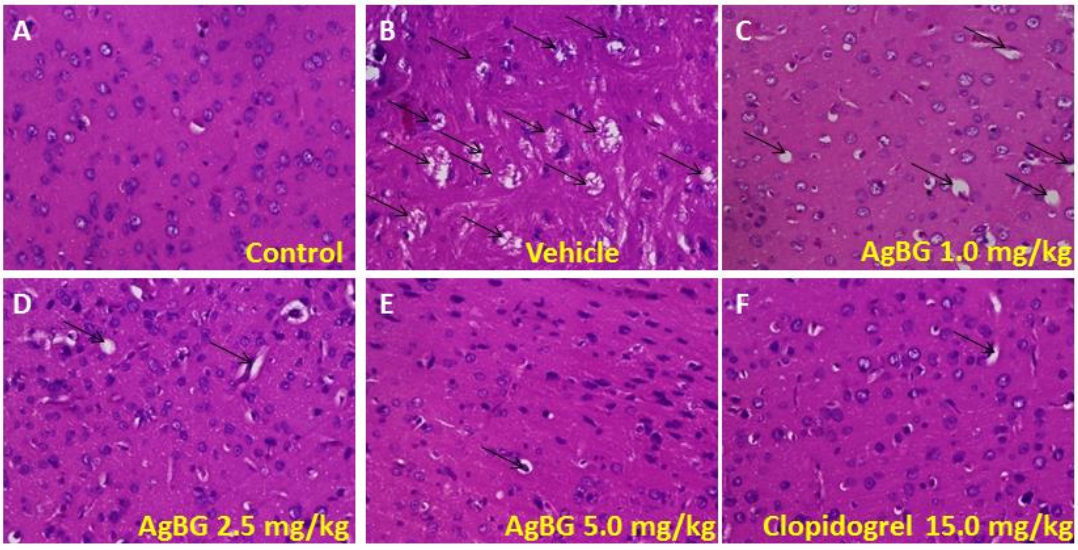

Figure 3.23 Effects of AgBG on cortex of the ischemic brain. (A-F) show the histology of different rat brain slices stained with (H.E., haematoxylin and eosin). A-control, B-vehicle, C-AgBG 1.0 mg/kg, D-AgBG 2.5 mg/kg, E-AgBG 5.0 mg/kg, and F-clopidogrel 15 mg/kg. Number of cells with vacuole in Figure 3.23 are followings A-control (1), B-vehicle (34), C-AgBG 1.0 mg/kg (12), D-AgBG 2.5 mg/kg (6), E-AgBG 5.0 mg/kg (3), and F-clopidogrel 15 mg/kg (2).The arrows indicate cavitation of the cytoplasm (n=3).

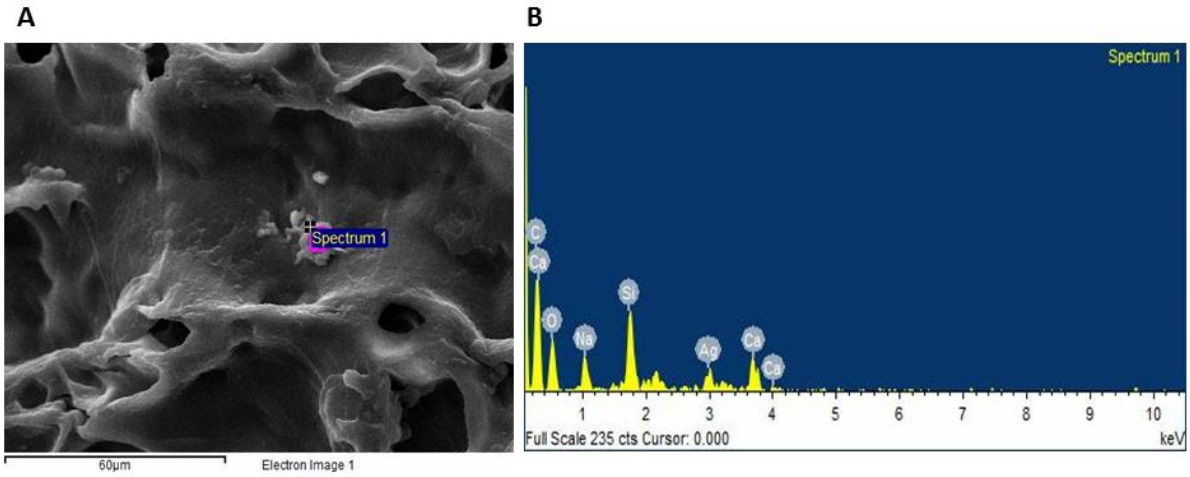

Figure 3.24 (A) SEM images and (B) Energy-dispersive X-ray spectroscopy of cortex of the ischemic brain at the dose of 5.0 mg/kg AgBG on the last day of the experiment.

3.5.9 VEGF levels

3.5.9.1 VEGF levels in BaBG study

Figure 3.25 shows that VEGF levels estimated using ELISA assay. VEGF was significantly increased in BaBG treated ischemic brains compared with vehicle group [$F(5, 29) = 37.03$; $P<0.05$] at 28 days after ischemia. The increase was found to be dose-dependent. Interestingly, treatment with clopidogrel did not exert any significant effect on VEGF levels compared to the vehicle group.

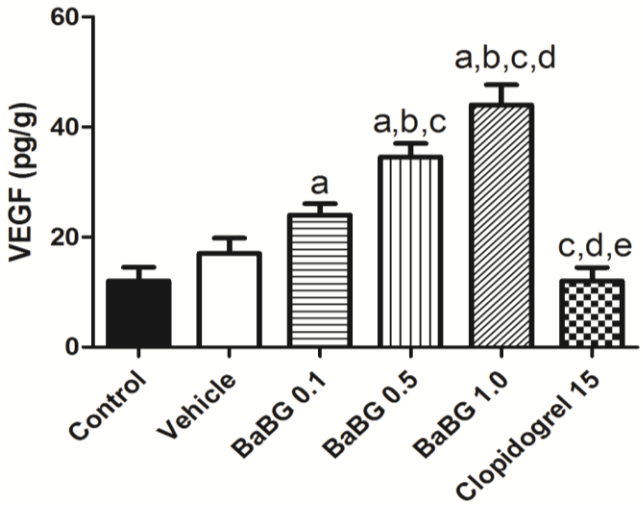

Figure 3.25 Bars represents the effects of BaBG on VEGF level in MCAO rats. All values are mean ± SEM (n=4). [a]$P<0.05$ compared to control group, [b]$P<0.05$ compared to vehicle group, [c]$P<0.05$ compared to BaBG 0.1, [d]$P<0.05$ compared to BaBG 0.5 and [e]$P<0.05$ compared to BaBG 1.0 [One-way ANOVA followed by Student Newman-Keuls post-hoc test].

3.5.9.2 VEGF levels in AgBG study

Figure 3.26 shows that VEGF levels, estimated using ELISA assay, were significantly increased in AgBG treated ischemic brains compared with vehicle group [F (5, 29) = 30.10; P<0.05] at 28 days after ischemia and the increases was found to be dose-dependent. Interestingly, treatment with clopidogrel did not exert any significant effect on VEGF levels compared to the vehicle group.

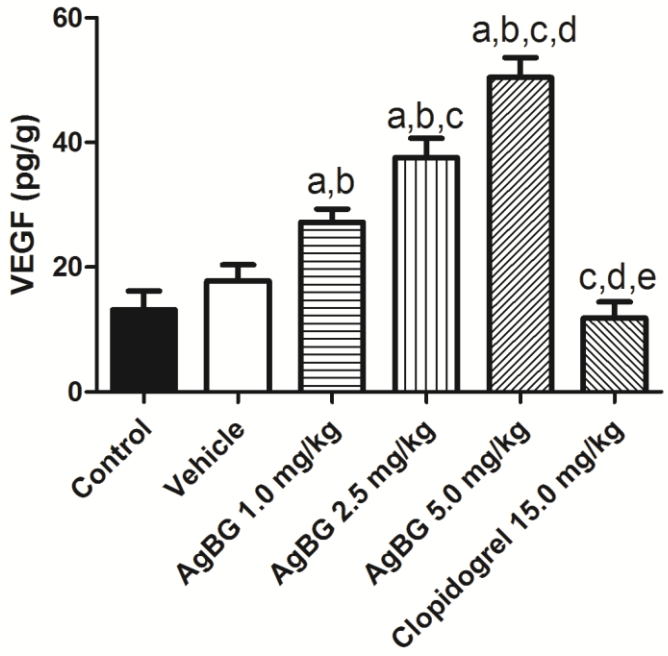

Figure 3.26 Bars represents the effects of AgBG on VEGF level in MCAO rats. All values are mean ± SEM (n=4). [a]P<0.05 compared to control group, [b]P<0.05 compared to vehicle group, [c]P<0.05 compared to AgBG 1.0, [d]P<0.05 compared to AgBG 2.5 and [e]P<0.05 compared to AgBG 5.0 [One-way ANOVA followed by Student Newman-Keuls post-hoc test].

3.6 Discussion

We have prepared, characterized and evaluated a parenteral micro-suspension of BaBG & AgBG for pharmacological treatment of cerebral ischemia possessing antiplatelet and antithrombotic activities. Treatment with BaBG & AgBG, immediately after reperfusion following a 2-hour focal ischemic insult recovered cerebral vascular hemodynamics, reduced infarct volume and improved functional brain activity.

Both BaBG & AgBG micro-suspension have trapezoidal shape microparticles with hydroxyapatite layer on their surface. The particle size of Both BaBG and AgBG micro suspension at all the three tested dose were found to be less the 5 µm. Highest dose of both the parenteral BaBG and AgBG microsuspension were having negligible hemolysis, therefore considered to be safe for parenteral use.

In ischemic stroke there is impaired sensorimotor and cognitive function (Belayev et al. 1996). Due to the loss of limb function after stroke, behavioral test mostly focus on motor and sensory tests (Schaar et al. 2010). The neuro-behavioral test was performed for the assessment of the degree of damage over a period of time. The aim of stroke treatment is the revival of these behavioral functions. BaBG & AgBG treatment at all three doses led to regain of behavioral functions lost within 14 days of treatment. A similar recovery in behavioral function was obsereved by other drugs in MCAO rats (Liu et al. 2014). The postural reflex test is used for the assessment of motor performance in MCAO rats (Bederson et al. 1986). Postural reflux score increased in MCAO rats and treatment with BaBG & AgBG in all three doses mitigated the reflux score. The limb placing test is commonly used to examine the hind and forelimb motor activity in MCAO rats (De et al. 1989). Treatment

with BaBG & AgBG significantly improved limb motor activity indicating improved motor performance.

Exploratory behavior of the rat is used to explore the neural basis of spatial and motor behavior. MCAO rats have shown asymmetry of forelimb usage in cylinder test which was consistent with an earlier study (Hua et al. 2002). Cylinder test is commonly used to measure the asymmetry of forelimb usage caused by MCAO (Liu et al. 2014; Hua et al. 2002). MCAO rats showed a significant decrease in usage of their impaired forelimb. BaBG & AgBG treatment significantly ameliorated MCAO induced impairment in forelimb which suggested that BaBG & AgBG facilitates the spontaneous forelimb use (Liu et al. 2014). The recovery in neurological deficits with BaBG & AgBG could be due to its effect on brain vascular hemodynamics.

Ischemic injury results due to the cessation of blood supply in the brain (Gou et al. 2010; Fahlenkamp et al. 2014; Weaver et al. 2015). Both BaBG & AgBG improved cerebral blood flow which was reduced in cerebral ischemic rats. Studies have shown that restoring the blood supply in the brain alleviates neurological impairment in the rats (Belayev et al. 2003; Li et al. 2014; Yamauchi et al. 2017). There was a sustained increase in CBF from 4 h after BaBG & AgBG administration in all the doses. A robust 40% of baseline increase in CBF and 38 % of baseline increase in CBF was observed at 4 h with the highest dose of BaBG (1.0 mg/kg) & AgBG (5.0 mg/kg) respectively. Several studies have reported that use of antiplatelet drugs leads to recovery of blood flow after 6 h post-reperfusion in the ischemic model (Belayev et al. 2008; Yamauchi et al. 2017). Similarly, in our present study, both BaBG & AgBG inhibited platelet aggregation. This may be the reason for CBF enhancement,

which occurs from 4 h after reperfusion with BaBG & AgBG treatment. To understand the molecular mechanism behind the effect of BaBG & AgBG on brain vascular hemodynamics, VEGF expression was studied. VEGF is associated with angiogenesis and vascular remodelling (Gandin et al. 2016). In the present study, the VEGF level is slightly increased in vehicle rats than the sham rats after 28 days of MCAO injury, which was consistent with the earlier report (Gandin et al. 2016). Both BaBG & AgBG dose-dependently increased VEGF in the ischemic brain. Increase in VEGF level has been reported to aid in the recovery of pathological as well as both neurological deficits in MCAO rodent model (Gandin et al. 2016).

Further, focal cerebral ischemia leads to activation and accumulation of platelet in brain-injured microvascular bed (Freedman et al. 2008; Abumiya et al. 2000). The injured vascular wall releases ADP, platelet aggregation agonist (Choudhri et al. 1998) which enhances thrombus formation even after reperfusion of the MCA and is responsible for the post-ischemic hypoperfusion and ongoing neuronal damage (Choudhri et al. 1998). Both BaBG & AgBG inhibited ADP-induced platelet aggregation both *in-vitro* and *ex-vivo*. Inhibition of ADP-induced platelet aggregation is reported to improve the brain functional activity, vascular hemodynamics and heal ischemic lesions (Marcus et al. 1997; Pinsky et al. 2002; Belayev et al. 2003 and Cha et al. 2008). Therefore, the observed recovery from MCAO induced brain deficits with BaBG & AgBG treatment could be due to the inhibition of ADP-induced platelet aggregation. Furthermore, BaBG & AgBG pretreatment shows antithrombotic action in vivo against carrageenan-induced rat tail thrombosis. The observed prophylactic anti-thrombotic activity of BaBG & AgBG could be due to their *ex-vivo* antiplatelet action. BaBG & AgBG inhibited ADP-induced platelet aggregation ex-vivo.

Similarly, SolCD39 (soluble form of Cluster of Differentiation 39) also known as ectonucleoside triphosphate diphosphohydrolase-1 inhibited ADP-induced platelet aggregation ex-vivo (Marcus et al. 1997; Pinsky et al. 2002) and improved the neurological score, postischemic cerebral perfusion and reduced total infarct volume (Belayev et al. 2003; Cha et al. 2008). Therefore, the observed recovery in neurological deficits and reduction in infarct volume with BaBG & AgBG treatment against MCAO induced injury could be due to the inhibition of ADP induced platelet aggregation.

Cerebral ischemic lesions induced after the reperfusion was confirmed and estimated by histology of the cortex of the ischemic brain. Histological examination shows the presence of multiple vacuolated interspaces and absence of intact neurons in MCAO group indicating brain injury in rats (Sarshoori et al. 2014). However, the corresponding areas of brain sections from the BaBG & AgBG treatment group showed intact neurons, indicating that the treatment was efficiently healing brain injury related to the cerebral ischemia.

Further, ischemic damage leads to the decline in the cortical electrical activity in the cortex of the brain. EEG is widely used as a parameter to assess electrical activity in cerebral ischemia and reperfusion injury as well as the assessment of drug effects (Martin et al. 1998 and Nicotera et al. 1999). We observed that BaBG & AgBG significantly improved the ischemia-induced reduction in EEG potential amplitude which indicated that BaBG & AgBG suspension induced significant functional recovery after cerebral ischemia-reperfusion injury.

Our results show that BaBG & AgBG recovered cerebral vascular hemodynamic, reduced infarct volume and improved functional brain activity. The pharmacological effect of BaBG & AgBG in the MCAO injury may be due to its antiplatelet activity and its ability to

stimulate VEGF level in the ischemic brain region. Further, BaBG & AgBG shows antithrombotic activity against carragenan rat tail model. Therefore, BaBG & AgBG may be a potential novel compound for the management of ischemic stroke.

In summary, we had prepared the penetrate able micro-suspension of the medicated bioactive material and evaluated its potential against cerebral ischemic-reperfusion injury in rats. BaBG & AgBG micro-suspension effectively ameliorates MCAO induced infraction and behavioral impairment, probably due to its ability to inhibit ADP-induced platelet aggregation and recovery of CBF due to VEGF stimulation. Further, it also normalize the cortical electrical activity, and functional brain activity as indicated by EEG. Moreover, BaBG & AgBG demonstrates prophylactic anti-thrombotic activity against carrageenan-induced thrombosis. Thus, BaBG & AgBG can be a potential compound for the treatment of cerebral ischemia.