CHAPTER 5

Nanocapsule mediated delivery of ginkgolide B combats cerebral ischemia reperfusion injury in young and aged rats
INTRODUCTION

Ischemic brain injury, a leading cause of death and adult disability worldwide, has drawn much attention of the drug development arena. Cerebral ischemia causes a reduction in oxygen supply to the brain and leads to energy failure by dropping the cellular ATP biosynthesis. Oxygen free radicals generated during post-ischemic reperfusion are a causative agent for damaging neuronal cells particularly mitochondria. (Siesjö et al, 2008)

Mitochondria are a major source of superoxide anion (O$_2^-$) and other reactive oxygen species (ROS) that may result from O$_2^-$ produced by the electron transport chain (Chan 2006) This ROS-induced damage alters the function of many metabolic enzymes in the mitochondrial matrix, as well as those comprising the electron transport chain. (Schriner et al, 2005) Thus mitochondria are both a major source of oxidants and a target for their damaging effects, and, therefore, mitochondrial oxidative stress appears to be a cause, rather than a consequence, of cell death.

Oxidative damage also accelerates with ageing and this contributes to the decreased oxidative phosphorylation of the inefficient mitochondria. (Toescu 2003) The situation becomes so severe that the cellular antioxidant system falls far short to balance the increased oxygen free radical pool in the cerebral tissue. Recently, it was demonstrated that Platelet Activating Factor (PAF) might contribute to the aggravation of ischemic cellular damage and might be a promoting factor of brain edema. (Pinckard et al, 1994)

In this state of affairs antioxidant supplementation as a drug as well as a scavenger seems to rescue from cerebral ischemia-reperfusion induced oxidative attack especially the mitochondria of aged population. Several studies have shown that antioxidants when applied exogenously are not capable of attenuating cerebral ischemia reperfusion injury (Forsman et al, 1988; Pereira et al, 1990; Chan 1998; Tasdeneroglu et al, 1993) Chemical compounds are almost ineffective in protecting neuronal cells from damage as Blood Brain Barrier (BBB) exists in between blood and brain interstitial fluid that restricts undesirable influx from the circulation into cerebral region. A basic BBB function is to eliminate the passage of macromolecules, microbial pathogens, and circulating leukocytes into the central nervous system. (Suzuki 1983)
Ginkgolides, isolated from the root, bark and leaves of *Ginkgo biloba* specifically inhibit platelet-activating factor from binding to its receptor thereby preventing platelet aggregation. This group of natural compounds has a long history of use in humans, lacks toxicity and is totally resistant to metabolism. Among them, Ginkgolide B (GB) is the most bioactive. Besides these benefits, this water insoluble compound has a low oral bioavailability and cannot extravasate the BBB due to its insoluble nature, a major challenge in CNS therapeutics. Nanocapsules have been well accepted in present age as potent drug delivery vesicles because of its ability to deliver polar and non polar compounds, nontoxic, biodegradability, nonimmunogenic, and sustained drug releasing ability in biological system. Nanocapsules have been reported to increase the oral bioavailability of poorly soluble compounds. (Ghosh et al, 2011; Win and Feng 2005)

Neuroprotective effect of GB intercalated in PLGA nanocapsules were evaluated against a BCCAO-reperfusion model in young and aged rat. We hypothesized that nanocapsulation of GB (NGB) followed by its oral administration would help the compound in better localization in brain and a sustained presence of GB would be capable of neutralizing the deleterious effects of ischemia-reperfusion injury.

MATERIALS AND METHODS

Materials

Poly(lactide-co-glycolide) (PLGA), Ginkgolide B, 2,6-dichloroindophenol (DCIP), Phenazine methosulfate (PMS), Succinic acid and Didodecyldimethylammoniumbromide (DMAB) were purchased from Aldrich (St. Louis, MO, USA) and Sigma (St Louis, MO, USA), respectively. Ethyl acetate (AR Grade) was purchased from Rankem Fine Chemicals (New Delhi, India). Chloroform and Methanol were purchased from E. Merck. QC was purchased from Sigma Chemicals (St. Louis, MO, USA). All other reagents were of analytical grade.

Preparation of Ginkgolide B (NGB) incorporated polylactide nanocapsules

A modified emulsion-diffusion-evaporation method was used to make NGB nanocapsules. In brief, PLGA was dissolved in 2.5 ml of ethyl acetate at room temperature. GB was dissolved in 1ml of ethyl acetate. The organic solution of PLGA and drug in ethyl acetate was then emulsified with 5 ml of an aqueous phase containing
didodecyldimethylammonium bromide (DMAB). The resulting o/w emulsion was stirred at room temperature for 3 h before homogenizing at 15,000 rpm for 5 min with a high-speed homogenizer (Polytron PT4000; Polytron Kinematica, Lucerne, Switzerland). The organic solvent was removed by constant stirring on a water bath set at 40 °C. The suspension was ultracentrifuged at 105,000 g in Sorval RC 5B Plus using the rotor Sorval T-865 for 1 h. The pellet of nanoparticles was washed with PBS twice and re-suspended in 2 ml PBS.

**Characterization of nanocapsules**

To estimate the intercalated drug in nanocapsules, the pellet was then dissolved in 2 ml of ethyl acetate and kept for 3 days at 4°C. The O.D. was measured at λmax (GB) 220nm and % of incorporation was calculated against a standard plot of GB.

Percent encapsulation was calculated using the following formula

\[
\text{Entrapment efficiency (EE)} = \frac{\text{Amount of GB present in the nanocapsules}}{\text{Amount of GB used to prepare nanocapsules}} \times 100
\]

Nanocapsules were observed under atomic force microscope for size and shape evaluation, surface charge and polydispersity index recorded in a Malvern Zetasizer Nano ZS instrument.

**Study design in animal model**

Male Wister rats of two age groups (2 months and 20 months) weighing 160–180 g and 415–440 g, respectively, were used. Experiments were done in animals with a prior approval of Animal Ethics Committee, India, Registration No. 147/99/CPC SEA. The animals were kept in a temperature and humidity-controlled housing with 12-h light and dark cycles. They were acclimatized for 3–5 days to the new environment before use and allowed free access to food and water. Rats from each category (2 months old and 20 months old) were subdivided into four groups of 20 animals. Two groups were used for saline treatment (one group for sham operated control and other group as untreated BCCAO-reperfused control). The remaining two experimental groups of rats were used for treatment with free GB, and nanocapsulated GB treatment respectively. Nanocapsulated GB (2mg/kg b.wt.), was fed orally 24 hrs before BCCAO. In control experiments, an identical dose of GB suspended in neutral oil was also fed orally 24 hrs before ischemic insult.
**Induction of cerebral ischemia**

Rats from both young and aged groups (excluding normal groups) were anaesthetized by a single intraperitoneal injection of urethan (17 mg/kg), were made ischemic by bilateral clamping of the common carotid arteries (BCCAO) for 30 min; normal blood flow was restored for next 30 min by withdrawing clamps. Body temperature of animals during whole period of experiment was maintained normal by keeping under filamental lamp (100 W). Sham operated animals were subjected to the same surgical procedure without BCCAO. (Ghosh et al, 2010) All experimental rats were killed by decapitation. The brain was isolated immediately and its surface was rinsed with ice-cold saline.

**Histological examination of hippocampal slices of experimental animals**

Hippocampal portions of experimental animals were removed quickly at the time of sacrifice and further fixed overnight in 4% paraformaldehyde at 4 °C. Postfixed portions were embedded in paraffin, and 5 μm sections were cut on a microtome. The sections were stained with cresyl violet and examined under a light microscope.

**Isolation of mitochondria from rat brain**

Dissected rat brain from experimental as well as sham operated animals were homogenized (10% w/v) in buffer (1M) containing 20mM Hepes-KOH, 10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM DTT, 0.1mM PMSF and standard Percoll gradient differential centrifugation method using Sorvall High Speed Centrifuge at 4° C. (Navarro and Boveris 2004)

**Measurement of ROS level in rat brain mitochondria**

Intracellular ROS level was measured in brain mitochondria (Betainder et al, 2002).

**Lipid peroxidation assay**

For the determination of conjugated diene method of Recknagel et al., 1984 was followed.

**Reduced glutathione (GSH) level in rat brain mitochondria**

Glutathione level in rat brain mitochondria was determined by using tetrachloroacetic acid with EDTA as protein precipitating reagent. (Davila et al 1991)

**Succinate Dehydrogenase (SDH) activity assay**

The enzyme was assayed as per the method of Elingold et al., 2008

**NADH oxidase activity assay**

NADH oxidase activity was measured as per the method of Elingold et al., 2008
Mitochondrial membrane potential

Mitochondrial membrane potential was measured by rhodamine 123 fluorescence. The reaction mixture contained: mitochondria (1-2 mg protein/ml), 0.5 mM ADP, 5 mM L-malate, 5 mM L-glutamate, 10 mM succinate and 3 μM rotenone in respiration buffer containing 240 mM sucrose, 34 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 9 mM HCl-Tris, and 6 mM Na₂HPO₄-KH₂PO₄ (pH 7.4). Rhodamine 123 (1 μM) was added to mitochondria at 37 °C and after 10 min incubation, the reaction mixture was centrifuged at 6800×g. Rhodamine 123 accumulation in mitochondria was determined by the difference between the fluorescence in the supernatant and in a solution of rhodamine 123 (1 μM). Fluorescence was measured in a Perkin Elmer LS3B spectrofluorometer, at 498 nm excitation and 525 nm emission. (Ghosh et al, 2011)

Quantitation of cerebral edema

Cerebral edema due to the entry of plasma water resulting into swollen brain of rats was expressed as a percentage of water content in whole brain of normal, sham operated as well as other experimental groups. Brain samples obtained after experiment were immediately weighed on an electronic balance (Sartorious VP61S) to obtain the wet weight. Brain samples were then dried at 100°C for 24 hours to obtain the dry weight and the tissue water content calculated as

\[ \frac{(W_{\text{wet}} - W_{\text{dry}}) \times 100}{W_{\text{wet}}} \]

where \( W_{\text{wet}} \) is the initial weight of brain tissue and \( W_{\text{dry}} \) is the dry weight of the same tissue.

Western blot analysis to detect iNOS in brain cytosolic fraction

Cytosolic fraction (30 μg total protein) of rat brain was subjected to 10% SDS/PAGE followed by electrophoretic transfer to PVDF membrane (Millipore) at 15 V for 20 min by using Semi dry (Bio-Rad) transblot apparatus. Membrane was blocked in 4% BSA in PBS (overnight) at 4°C, followed by incubation with the primary rabbit anti iNOS antibody (1:1000) for 3 h and then in secondary alkaline phosphatase conjugated anti rabbit goat IgG antibody (1:2000) for 1 h 30 min. Bands were visualized by the development of colour using Sigma premixed BCIP/NBT substrate solution. Their density was analysed with Image J software system.
In situ apoptosis detection assay

Formalin fixed, paraffin embedded glass slide mounted brain sections of experimental rats were stained with Anti-BrdU; FITC antibody using the Apo-BrdU In situ DNA fragmentation assay kit (Biovision K401-60) as per the manufacturer’s protocol.

Statistical analysis

The mean and standard deviation were calculated for all data. Significant differences between means were evaluated by an analysis of variances. A difference was considered significant when p<0.05.

RESULTS

Nanocapsules characterization

The physicochemical characteristics like particle size, polydispersity index, drug encapsulation efficiency and zeta potential of NGB are summarized in the following Table.

Table 1. Nanocapsule characterization

<table>
<thead>
<tr>
<th>Type of Particle</th>
<th>EE</th>
<th>Size</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGB</td>
<td>68%</td>
<td>39nm</td>
<td>0.251</td>
<td>72</td>
</tr>
</tbody>
</table>
Fig. 1. Atomic Force Microscopic images of GB nanocapsules

Neuroprotective effect of QC nanocapsules on hippocampus neurons against 30 minutes BCCAO-reperfusion

Histological study of the hippocampal neurons using cresyl violet staining show normal pyramidal cells with round and pale stained nuclei in sham operated control young rats, whereas aged sham operated control rats show lesser number of cells. Thirty min BCCAO and 30 min reperfusion induced significant cell loss is observed in aged animals. Young ischemia reperfused animals show neuronal loss, shrinkage, and dark staining of neurons. GB pretreated animals after ischemia reperfusion show severe pyramidal cell loss with no noteworthy improvement than ischemia reperfused group. NGB pretreated groups show only scattered ischemic neurons with visibly less pyknosis and cell shrinkage. (Fig.2).
Fig. 2. Representative photomicrographs of cresyl violet stained hippocampal sections of experimental rat brain. Sham operated control (Sham) young rats show normal pyramidal cells with round and pale stained nuclei, whereas aged Sham rats have many dying or dead cells with pyknotic nuclei. 30 min BCCA0 and 30 min reperfusion (Isch) induced extensive cell loss is observed in aged animals. Young Isch animals show neuronal loss, shrinkage, and dark staining of neurons. GB pretreated animals (GB + Isch) show ischemic neuronal loss. NGB pretreated (GB + Isch) show organized neurons with visibly less pyknosis and cell shrinkage.
Impact of NGB on age-related diene generation in brain tissue due to induction of 30 minutes BCCAO-reperfusion

Lipid peroxidation is a prime marker for stress induced membrane damage. Conjugated diene is formed as a result of lipid peroxidation. In young rat brain mitochondria ischemia-reperfusion caused a substantial increase in the diene level (Table 1). In aged rats also, cerebral ischemia and reperfusion cause a massive increase in the diene level in brain mitochondrial bilayer. A significantly high level of lipohydroperoxide in aged sham operated rats than in young rat brain was also noted here. Pretreatment of rats with GB 24 h prior to ischemic insult resulted in no significant protection against global transient cerebral ischemia and reperfusion-mediated diene generation in brain both in young and aged animals. But oral application of NGB in the same situation could arrest diene formation in young and aged rat brain mitochondrial membrane.

Table 2. Effect of NGB on age related formation of conjugated diene and GSH in rat brain mitochondria by the induction of 30 minutes BCCAO-reperfusion

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Diene level (mumoles of conjugated diene/mg protein)</th>
<th>Reduced glutathione (mumoles of GSH/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Aged</td>
</tr>
<tr>
<td>Sham Control</td>
<td>5.65±0.09</td>
<td>9.03±0.12</td>
</tr>
<tr>
<td>Cerebral ischemia-reperfusion (ISCH)</td>
<td>10.34±0.34</td>
<td>12.59±0.10</td>
</tr>
<tr>
<td>GB + ISCH</td>
<td>9.35±0.11</td>
<td>12.00±0.14</td>
</tr>
<tr>
<td>NGB + ISCH</td>
<td>6.75±0.16</td>
<td>11.42±0.08</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. Cerebral ischemia-reperfusion group was compared with normal and the value was significantly different *P<0.01 Experimental groups (GB and NGB) were also compared with ischemia reperfused group (ISCH) and the value was significantly different **P<0.05.
Effect of NQC on 30 minutes BCCAO-reperfusion on antioxidant defense in young and aged rat brain mitochondria

Glutathione, the most important intracellular antioxidant, was found significantly depleted in brain mitochondria of young and aged rats after cerebral ischemia-reperfusion (Table 2). GSH level was lower in sham operated aged animals compared to young. The level was significantly more towards the sham control level in groups of young and aged rats that received NGB prior to ischemia, whereas 'free' GB resulted in no significant effect.

Table 3. Effect of NGB on age related ROS generation, SDH and NADH oxidase activity in rat brain mitochondria by 30 minutes BCCAO-reperfusion

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>DCF fluorescence of normal</th>
<th>Rate of SDH</th>
<th>NADH oxidase (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100±6.5</td>
<td>1.5±0.12</td>
<td>5.65±0.09</td>
</tr>
<tr>
<td>Cerebral ischemia</td>
<td>103±5.7</td>
<td>0.95±0.10</td>
<td>9.03±0.12</td>
</tr>
<tr>
<td>reperfusion (ISCH)</td>
<td>300±14.6</td>
<td>0.41±0.02</td>
<td>12.59±0.10</td>
</tr>
<tr>
<td>GB + ISCH</td>
<td>259±11.0</td>
<td>0.72±0.03</td>
<td>9.83±0.16</td>
</tr>
<tr>
<td>NGB + ISCH</td>
<td>153±7.0</td>
<td>0.90±0.08</td>
<td>11.78±0.15</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE. Cerebral ischemia-reperfusion group was compared with normal and the value was significantly different *P<0.01 Experimental groups (GB, NGB) was also compared with ischemia reperfused group (ISCH) and the value was significantly different *P<0.05 **P<0.01

ROS generation and mitochondrial respiratory enzyme complex activities due to 30 minutes BCCAO-reperfusion

Mitochondrial ROS level was found to increase with respect to sham by the induction of 30 minutes BCCAO-reperfusion in young and aged rats. The ROS values for sham operated control young rats were taken as the basal value. The Relative Fluorescence intensity (RFI) was higher in sham aged rats. NGB was most potent in controlling BCCAO-reperfusion induced ROS generation in brain cell mitochondria. (Table 3). The mitochondrial respiratory complex I has a NADH oxidase activity while the complex II is succinate dehydrogenase. Aged rats showed a decreased activity of these enzymes than
their young counterparts. Rats subjected to ischemia reperfusion insult showed a significant increase in NADH oxidase activity with a simultaneous decrease in SDH activity than the sham operated control values. (Table 3) Only GB treatment was unable to bring the levels close to the control values. However these levels were found to be most close to the sham operated control values when young and old rats were fed with a single dose of NGB 24h prior to ischemia.

**Mitochondrial membrane potential dissipation due to 30 minutes BCCAO-reperfusion and effect of NGB pretreatment**

The effect of 30 minutes BCCAO-reperfusion on mitochondrial membrane potential was investigated in rat brain mitochondria. Mitochondrial membrane potential was found to decrease sharply due to 30 min of BCCAO-reperfusion in young and aged rats. (Fig 3) On the other hand animals pretreated with NGB could cerebral ischemia reperfusion induced dissipation of membrane potential.

![Membrane potential of mitochondria isolated from experimental rat brain cerebral ischemia reperfused groups (Isch) groups were compared with sham control animals (Sham) and drug treated groups were compared with Isch group. Values are mean ± SEM for 5 rats. *indicates p<0.001.](image)

**Age related change in cerebral tissue water content induced by ischemia and reperfusion**

The effect of ischemia-reperfusion on brain edema was assessed. Cerebral ischemia reperfusion caused a marked increase in the water content of brain of young and aged animals.(5 and 6 % respectively) But all animals pretreated with NGB got significant protection (P< 0.05) from entry of plasma water into brain. The amelioration of cerebral
ischemia induced edema formation with NGB was associated with reduced accumulation of plasma water in brain. (Fig 4)

**Fig. 4.** Percentage water content in brain of normal (NOR), Sham operated control (SHAM), 30 min cerebral ischemia and subsequent 30 min reperfusion (ISCH), GB pretreated ISCH (GB+ISCH), NGB pretreated ISCH (NQC+ ISCH), in both young and aged rats. Values are mean ± SEM for 5 rats. * * indicates p<0.01 significantly different from normal, ** indicates p<0.01 significantly different from ISCH groups.

**Fig. 5.** Western Blot analysis of iNOS protein expression in cytosolic fraction of experimental rat brain. Histogram showing representative pixel intensities (arbitrary units of densitometric analysis using Image J software) of the immunoblot performed with different individual rats.
iNOS expression in cytosol

iNOS immunoreactivity was evident as a band in brain cytosol fractions in young and aged rats. (Fig5). A strong iNOS expression was evident in BCCAO-reperfusion group. However NGB pretreatment could check iNOS expression in both young and aged rats.

DNA fragmentation assay

We observed increased BrdU positive cells indicating apoptosis in brain of BCCAO-reperfused control young and aged animals in comparison to the sham control group. (Fig 6) NGB pretreatment showed visibly less apoptotic cells in brain hippocampal sections of both young and aged animals when compared to BCCAO-reperfusion group.

![Fig. 6. Representative photomicrographs of BrdU positive cells observed by double staining with BrdU (under FITC filter) and PI stained brain hippocampal sections of experimental animals. (10X) ISCH refers to rats subjected to 30 min moderate global cerebral ischemia by BCCAO followed by 30 min reperfusion.](image)
DISCUSSION

Bilateral Common Carotid Artery Occlusion (BCCAO) in rat is a unique model to study cerebral hypoperfusion associated neurodegeneration. This representation creates cognitive decline in animals and facilitates the study on the progression of neurodegeneration and cell death. (Farkas et al., 2007) A lot of studies have been undertaken using the model of permanent 2 vessel occlusion method (Ohtaki et al., 2006; Farkas et al., 2006; Shang et al., 2005) and has shown species and strain specific neurologic outcome in terms of tissue damage. But this method does not induce reperfusion oxidative injury, commonly observed in cases of stroke insult. A case of 30 min BCCAO followed by 30 min reperfusion in Wister rats produced oxidative stress similar to stoke type damage, caused cerebral edema along with an induction of BBB permeability. (Sinha et al., 2001; Sarkar et al., 2006; Das et al., 2008) In our study NGB was found effective in controlling cerebral ischemia reperfusion induced secondary ROS production, lipid peroxidation and depletion of endogenous antioxidants like GSH in neuronal mitochondria of young as well as aged rats. Apart from age related decline, cerebral ischemia reperfusion also showed serious deleterious effects on the respiratory complexes in mitochondria. GB in PLGA encapsulated nanoparticulated form was found potent to exert neuroprotective impact on cerebral ischemia reperfused rat brain mitochondria, irrespective of age. Animals receiving oral NGB (2mg GB/kg) demonstrated BBB integrity, thereby preventing edema, reduced the level of ROS, lipid peroxidation, depletion of endogenous antioxidants like GSH in neuronal mitochondria as a result of reperfusion, and protected mitochondria from dysfunction. NGB also could downregulate iNOS a key mediator of inflammation, generated during post ischemic reperfusion and protected neurons from undergoing apoptosis.

Neuronal cell loss especially the hippocampal CA1 neurons are worst affected in acute cases of cerebral ischemia reperfusion injury as well as in chronic ones like senile dementia of Alzheimer type. However BCCAO induced cerebral hypoperfusion does not cause severe cell loss in its early stages. (Farkas et al 2007) Hippocampal cell loss observed in our experiment (Fig 2) of 30 min BCCAO with subsequent 30 min reperfusion in aged as well as young rats might be due to the delayed neuronal death during reperfusion oxidative injury, a feature not occurring in cases of cerebral hypoperfusion by permanent 2 vessel occlusion method. Pyknotic nuclei, a sign of necrosis, observed in Fig 2 might have been produced during the BCCAO causing rapid
depletion of ATP and respiratory substrate while the cell loss might be the effect of reperfusion. NGB but not free GB pretreatment was able to protect pyramidal cells from such damage.

Cerebral ischemia causes a reduction in oxygen supply in brain along with depletion of glucose and ATP level and initiate tissue degeneration therein. During the ischemic phase, obstruction of blood flow results in ATP depletion and necrosis. (Sarkar and Das, 2006) Reperfusion corresponds to a sudden burst of oxygen flow which creates oxidative stress in brain. Biochemical effects commonly noticed following cerebral ischemia reperfusion include uncontrolled leakage of ions across the cell membrane, membrane depolarization and release of neurotransmitters glutamate and dopamine (Horst and Postigio, 1997). Excess glutamate release and stimulation of its receptors result in phospholipase activation, phospholipids hydrolysis and arachidonic acid release, ultimately, leading to apoptotic and necrotic cell death. In this context, mitochondria are highly vulnerable and play a predominant role in cell signaling leading cells from life to death. Overproduction of ROS generation due to rapid $O_2$ flow in brain mitochondria and decreased detoxification, destroy mitochondria. Oxidative damage in conjunction with calcium loading also leads to induction of mitochondrial permeability transition, leading to nonspecific pore in the inner mitochondrial membrane rendering the mitochondrial inner membrane permeable to solutes smaller than 1.5 kDa thus preventing oxidative phosphorylation. (Friberg et al, 2002). Increased ROS production in BCCAO-reperfused rat brain caused lipid peroxidation in mitochondria membrane (Table 2). Overproduction of lipid peroxides and aldehyde products can cause depletion of GSH through detoxification by GSH-Px and glutathione S-transferase. This is in conjunction with our findings. The post ischemic brain has a redox imbalance reflected from the activity of mitochondrial respiratory chain components, (Table 3). Net production of ROS at distal ends of the ETC (Fiskum et al, 2004) caused a depolarization of mitochondrial membrane leading to a fall in its potential as seen in the rhodamine 123 stained images (Fig 3) and formation of pores in the outer membrane (Starkov et al, 2004). The rate of generation of ROS in mitochondria increases gradually with ageing. Decreased endogenous antioxidant defences with age may further accelerate the cerebral ischemia reperfusion oxidative injury (Table-2) In pathogenesis as in ischemia- reperfusion neuronal cell mitochondria are unable to counter the elevated ROS level and succumb to irreversible damage.
Neurons in an aging brain are more vulnerable to stress (Davis, 1995) and might respond exaggeratedly in experimental models of stroke.

The formation of fenestrations in the BBB endothelial tight junction with a size of 100 nm as a consequence of ischemic injury, allowing the passage of macromolecules that are normally unable to reach the brain is a major pathophysiologic outcome. The hyperpermeability of the BBB during ischemia has been demonstrated by Ito et al., 1990. They also reported that only fat soluble molecules cross the BBB by transcellular diffusion. Preston et al., 2001 demonstrated BBB opening during ischemia in rat brain. It was also supported by our earlier work (Sinha et al, 2001) Cerebral ischemia resulted in a significant increase in brain cell water content (Fig 4). This edema development is a measure of uncontrolled entry of plasma water in the cells and a loss of BBB integrity (Lee et al, 2004; Pillai et al, 2009).

Changes in the antioxidant status of brain tissue during ischemia and reperfusion influence the extent of postischemic brain damage. Endogenous antioxidant levels in brain tissue are reduced during ischemia and remain below the baseline values until several days after reperfusion (Choi-Kwon et al, 2004). NGB showed free radical scavenging activity and hence was able to decrease the ROS level in rat brain mitochondria. The decreased ROS must be able to counterbalance the increased conjugated diene formation and depleted reduced glutathione. ROS mediated damage to the ETC was also taken care of by NGB where free GB could not protect the cells from oxidative damage. GB being hydrophobic was unable to enter the cell as such. Moreover these types of compounds are poorly absorbed through the GI tract (Scalbert et al, 2000). BBB penetration is the most important challenge in treating neurodegenerative diseases. Polymeric nanoparticles can cross the BBB and can deliver the therapeutics efficiently to the CNS (Reddy and Labhasetwar, 2009). The protective effect of NGBs could be due to the direct radical scavenging effect of GBs released from the nanocapsules localized in the brain. GB, a PAF antagonist could have prevented phospholipase activation and arachidonic acid formation. Oxidative metabolism of arachidonic acid generates ROS. Again protecting the BBB integrity prevented edema formation in brain and subsequent inflammatory signaling cascade activation. Thus iNOS level was found to be downregulated by NGB treatment. Since the BBB integrity was maintained, the NGBs localized in the endothelium of the cerebral vasculature could have protected it from ROS-induced damage. The NGBs localized in the brain may have protected the neurons, which are very
susceptible to damage due to ROS because neurons are rich in oxidation-prone, polyunsaturated fatty acids (Margaill et al, 2005).

Thus, encapsulation of GB in nanocapsules could have facilitated the intracellular delivery of the compound following their localization in the brain and might have neutralized the effect of the ROS generated both in the intracellular and mitochondrial environments, resulting in significant reduction in neuronal apoptosis (Fig.6) The damaging effects of ischemic-reperfusion injury can extend beyond the ischemic area because of systemic inflammatory response in stroke condition (Collard and Gelman, 2001) The conducive conditions created in the brain as a result of the sustained presence of GB prevented triggering of inflammatory cascade, (Fig 5) and its antiapoptotic mechanisms might play a role in facilitating the process of angiogenesis and neurogenesis in the infarcted brain. (Yasui and Baba, 2006) Thus, NGB may demonstrate their protective efficacy in ischemia-reperfusion injury via different pathways.

This experiment of using GB in polymeric nanocapsules and evaluating its efficacy in conferring protection to neuronal mitochondria in experimental model of stroke in both young and aged rats provide a way in the formulation of safe therapeutics that cross the BBB, intact or hyperpermeable, making it suitable for use in acute as well as chronic cases of neurodegeneration. A similar therapeutic strategy can also be developed to rescue other organs, such as heart, kidney, and liver, from the ischemia-reperfusion-induced damage caused by ROS. Thus, NGBs could be explored for therapeutic applications in several oxidative stress conditions.