CHAPTER 10	MICELLE NEUROPROTECTIVE AND TARGETING EFFICIENCY

10.1 Introduction

There are numerous strategies developed to overcome the cerebral ischemia. Among which hypothermia is commonly employed, however; the mechanisms of the neuroprotective effect are complex and not fully understood, but are believed to include several factors such as suppression of the neuro excitatory cascade and decrease of the amounts of free radicals (1). Cerebral metabolism decreases with a reduction in temperature. For every degree Celsius between 37 and 27°C, cerebral metabolism is decreased with approximately 8% (2). However, the beneficial effects of mild hypothermia could not be due to a decrease in metabolism alone (3). Hypothermia attenuates the formation of ROS during reperfusion after global ischemia. There is also a decrease in the lipid peroxidation during hypothermia compared to normothermia, which is obtained through higher levels of free radical scavengers (4). Glutamate production is decreased during hypothermia after middle cerebral artery occlusion (MCAO). There is also a decrease of glutamate production after global ischemia in hypothermic rabbits compared to normothermic rabbits (5). Induced apoptosis in neurons *in vitro* is suppressed by hypothermia. After ischemia, caspase activity, which mediates apoptosis, was decreased by hypothermia treatment in rats (6).

Although beneficial for survival and outcome, hypothermia treatment has made the early prediction of outcome more difficult. Neither the biomarkers S-100B and NSE (7) nor clinical signs can be used in the same manner as for patients not treated with hypothermia. Data on outcome prediction after hypothermia treatment are also sparse.

Initially cerebral ischemic stroke trials were carried out in higher species. But due to availability problem, cost and acceptability from ethical perspectives of small animals, animals such as the rat and the mouse are used. In most of cases stroke animal studies rat is used because of its close resemblance between humans cerebrovascular anatomy and physiology (8), its ease of handling, monitoring physiological parameters, ease of induction of cerebral ischemia (9) and the comparative similarity within strains. The selective MCAO model, which provides higher ischemic lesion growth size within 24 h, may be advantageous for studies of neuroprotective strategies (10).

As number of times it was observed that many drugs proves to be effective in smaller animals and fails in human clinical trials. Hence, it is recommended that once positive results are observed in small animals, the study should be later on replicated in higher species before proceeding to clinical trials (11).

10.2 Methods

Healthy adult male rats weighing 200–250 g were selected for the surgery. Since Nicergoline is a neuroprotectant which may affect the intensity of infarction (12), males are preferable due to their robustness. All the animals were fasted overnight before the experiment. Three rats are housed per cage. The bedding is to be changed regularly otherwise the animal are expected to eat whatever they find for example feces and even tail and skin of other animals. Therefore, animals were fed with standard laboratory rat chow and provided water *ad libitum*. However, 12 hr before surgery the food should be withdrawn and water is allowed *ad libitum*. The study was carried out in accordance with Institutional Animal Ethics Committee Pharmacy Department approved Protocol no: MSU/PAHRM/IAEC/2011/16.

10.2.1 Induction of transient global ischemia in brain

In accordance to the CPCSEA guidelines, animals were anesthetized with administrations of ketamine (100 mg/kg, I.P.) and diazepam (5 mg/kg, I.M.) respectively, and maintained with additional I.P. doses of ketamine (25 mg/kg) administered as necessary.

Several animal models have been developed in rats, mice and gerbils for the study of ischemic, hypoxic and hypoxic-ischemic brain injury (16). Global cerebral ischemia in rat was induced by two vessel carotid artery occlusion model. It involves occlusion of bilateral common carotid arteries using aneurysm clips which induces delayed neuronal death in animals (16) (17). The animals were divided into four groups each consisting six animals. Sham operated/control group animals were treated with saline. The animals were anaesthetized and the rectal temperature monitored with a digital thermometer inserted into the anus, was maintained at 37°C to 38°C throughout the operation by placing the animals under a heat lamp and warming them with a blanket. A midline cervical incision was made and the right common carotid artery was surgically exposed (18). Taking care not to damage the vagus nerve, the right common carotid artery was isolated using silk thread. After stabilization of 5 minutes, the right common carotid

artery was occluded with a sterile stainless steel bulldog clamp for 120 minutes to induce ischemia and was confirmed by visually observing blockage of blood flow. Subsequently, the clamp was removed and reperfusion was confirmed visually before the incision was sutured. The animal was allowed to recover and neomycin sulphate powder was sprinkled on the wound to prevent occurrence of infection. During the surgery and after the surgery, the animal was kept under halogen lamp to maintain body temperature until it started moving freely.

10.2.2 Procedure for Sham Surgery

For sham surgery, carotid arteries were not occluded and only exposed for the surgical period. The surgical period and anesthesia period was kept same as that for the test animal.

10.2.3 Evaluation of anti-ischemic activity

The neuroprotective of nicergoline loaded peptide conjugated micelle (NGPCM) and non-conjugated micelle (NGM) was studied by intranasal administration of micelle after 30 minutes of initiating transient global ischemia. During intra nasal administration anesthetized rats were placed on their backs, and a total of 20μL solution per rat (10μL in each nostril), saline and stroke homing peptide conjugated and non-conjugated micelle, was given intranasally using micropipette (1μL to10μL) attached with low-density polyethylene tubing, having 0.1mm internal diameter at the delivery site (19). The neuroprotective activity of conjugated and non-conjugated was evaluated with respect to brain infarct volumes, reduction in brain oedema expressed as the ratio of hemisphere areas and brain glutathione levels.

Group I: Surgical exposure of common carotid artery was done. Served as sham control.

Group II: Surgical activity + occlusion of carotid artery and intranasal administration of saline

Group III: Ischemic rat treated with non-conjugated nicergoline loaded micelle administered via intranasal route (NGM)

Group IV: Ischemic rat treated with stroke homing peptide conjugated nicergoline loaded micelle administered via intranasal route (NGPC).

10.2.4 Histological estimation of brain infarct volume

After 24 hr of reperfusion the animals were euthanised with pentobarbital (100 mg/kg). The brains were quickly removed, washed with ice-cold Normal Saline containing 0.9% sodium chloride, blotted and freezed at -70^{0}C for 4-5 hrs. Immediately the brain was coronally sliced (2-mm-thick) with fresh chromium blade and the slices incubated for 30 min at 37°C with TTC solution. All the slices with the caudal face upward were recorded with a digital camera, the images transferred to a computer (**Figure 10.1**) and analyzed using an image analyzer (Image J, NIH, USA). Total infarct volume was the sum of the infarct volumes from all of the slices. To correct for brain oedema in lesion, ischemic hemisphere, we determined infarct volume, expressed as a percentage of control hemisphere volume, in each rat by the following formula and tabulated in **Table 10.1** and shown graphically in **Figure 10.3** (18). Brain oedema was evaluated by ratio of ischemic/non-ischemic hemispheres.

$$Infarct\ volume\ (\%) = \frac{[LV - (RV - MV)]}{LV} \times 100$$

10.2.5 Preparation of TTC

2,3,5-triphenyltetrazolium chloride (TTC) solution (2%w/v) was prepared in phosphate buffer pH 7.4 immediately before use TTC being light sensitive and was masked with aluminum foil till used further.

10.2.6 Estimation of brain glutathione

Glutathione (GSH) is key factor involved in the detoxification of electrophilic metabolites and reactive oxygen intermediates (20) and as a co-substrate of glutathione peroxidase, it plays an essential role in protection from free radicals (21). Hence, glutathione level in the brain was taken as the index for the antioxidant activity of nicergoline (22).

GSH in the samples was determined by the method developed by Moran et al (23). After 22 hr of reperfusion animals were euthanised with pentobarbital (100 mg/kg). The brains were quickly removed and homogenized. To 1 mL brain homogenate 1mL trichloroacetic acid solution (chilled) was added and centrifuged at 2500 rpm for 10min. To 0.5mL of resulting supernatant 4mL DTNB solution and 1.5mL phosphate buffer were added and the absorbance recorded at

412nm. To prepare blank instead of tissue homogenate distilled water was taken and was given the same treatment as that of tissue homogenate samples. The GSH level was estimated using formula given below and then expressed in micrograms per gram of brain tissue and tabulated in **Table 10.1** and shown graphically in **Figure 10.4**.

x = (y-0.0046)/0.0034

Where, x = GSH level in μg/mL

y = absorbance.

Trichloroacetic acid solution (10%w/v):- Dissolve 10g trichloroacetic acid in 80 ml distilled water and make up the volume up to 100 ml with distilled water.

DTNB (dithio-bis-nitrobenzene) solution (0.6 mM):- Dissolve 60 mg of DTNB in 100 ml of 1%w/v sodium citrate solution.

Phosphate buffer (0.2 M, pH 8):- Mix 25ml of 2.72%w/v solution of potassium dihydrogen phosphate with 23ml of 0.8%w/v solution of sodium hydroxide and make up the final volume up to 100 ml with distilled water.

10.2.7 Method to prepare brain homogenate

Brain (whole) was isolated carefully in ice-cold saline. The organ was then blotted and weighed (wet mass) and suspended in 0.25M sucrose solution (chilled) and quickly blotted on a filter paper. The tissue was then minced and homogenized in 10 volumes of chilled 10mM Tris-hydrochloride buffer with 20 strokes of Teflon pestle in glass homogenizer at a speed of 2500rpm. The homogenate was then centrifuged at 10,000rpm at 4C for 15 min using Sigma centrifuge. The clear supernatant was used for the estimations.

Sucrose solution (0.25 M):- 85.58gm of sucrose was dissolved in 200ml distilled water and diluted to 1000ml with distilled water.

Tris-hydrochloride buffer (10mM, pH 7.4):- 1.21gm Tris was dissolved in 900ml distilled water and the pH adjusted to 7.4 with 1M hydrochloric acid. The resulting solution was diluted to 1000ml with distilled water.

10.2.8 Preparation of coumarin loaded peptide conjugated TPGS/PF 127 micelles

Coumarin micelles were prepared in same manner as explained for Nicergoline micelle in chapter 4. In brief, 0.1 mg of Coumarin, 10 mg PF 127 and 25 mg TPGS were dissolved in chloroform : methanol mixture. A thin film was formed by evaporating organic solvent using rotary evaporator at 40°C. This film was then kept in desiccators under vacuum for 24 hrs further to remove any remaining organic solvent. Then thin film of Coumarin was dispersed in HEPESS buffer pH 7.4. The unincorporated coumarin was separated by centrifugation at 5000 rpm and 4°C. The prepared coumarin micelles were characterized for size and zeta potential. Coumarin loaded immunomicelle were prepared by same method as described in chapter 6.

10.2.8.1 Targeting efficiency of peptide conjugated micelle

In an ischemia induced rat Coumarin entrapped non-conjugated and CLEVSRKNC peptide conjugated micelles i.e immunomicelle 20 µl were administered into each nostril.

Group I: Coumarin entrapped non conjugated micelle

Group II: Peptide conjugated Coumarin entrapped micelle

After 1 hr of administration of coumarin loaded micelle animals were euthanized with pentobarbital (100 mg/kg). The brains were quickly removed, washed with ice-cold Normal Saline containing 0.9% sodium chloride, blotted and. The brain were sectioned into 2 mm thickness sections and stained with TTC as mentioned above. The TTC stained sections were fixed in 4% paraformaldehyde for 48 hr and frozen at −70 °C. The frozen 2 mm brains sections showing ischemic zone and non-ischemic zone were cut into 20 µm thicknesses sections using cryotom Cryostat. The sections were washed twice with PBS (pH 7.4) and immediately examined under confocal microscope.

10.3 Statistical Analysis

The data are presented ± SD, and differences between the groups were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's post-test for multiple means comparison or Student's t test. Differences greater at $P < 0.05$ were considered insignificant.

10.4 Results

Table 10.1 Brain infarct volume, ratio of hemisphere areas, glutathione estimated for anti-ischemic activity

	Sham control (Group I)	Treated		
		Control (ischemic, Group II)	Non conjugated Nicergoline Micelle NGM (i.n.; Group III)	Peptide conjugated Nicergoline Micelle NCPG (i.n.; Group IV)
Number of Animals (N)	3	3	3	3
Infarct volume (mm^3)	---	20.5 ± 3.2	15.3 ± 2.28	21.5 ± 2.62
Brain GSH (µg/g of tissue)	90.3 ± 5.3	75.5 ± 4.1	98.3 ± 4.2	100.2 ± 4.2

Values are expressed as mean ± SD, n=3.

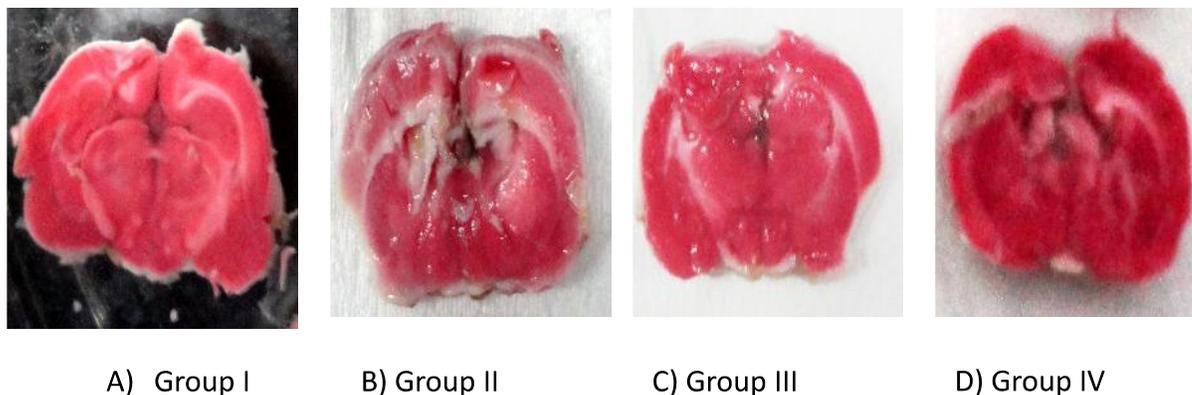A) Group I   B) Group II   C) Group III   D) Group IV

Figure 10.1 TTC stained brain slices A Sham control (Group I), B Ischemic control (Group II), C NGM (Group III), D NGPC (Group IV)
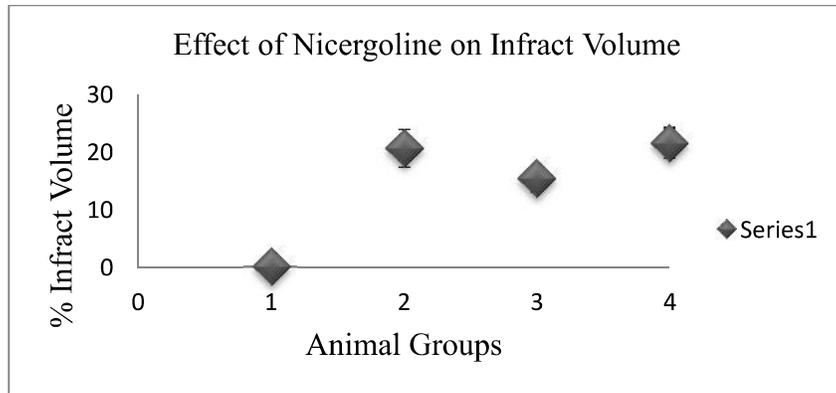
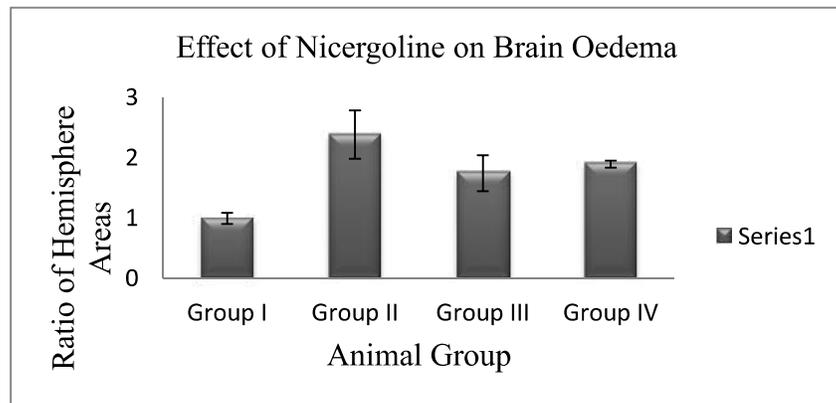**Figure 10.2** Effect of NG on infarct volume

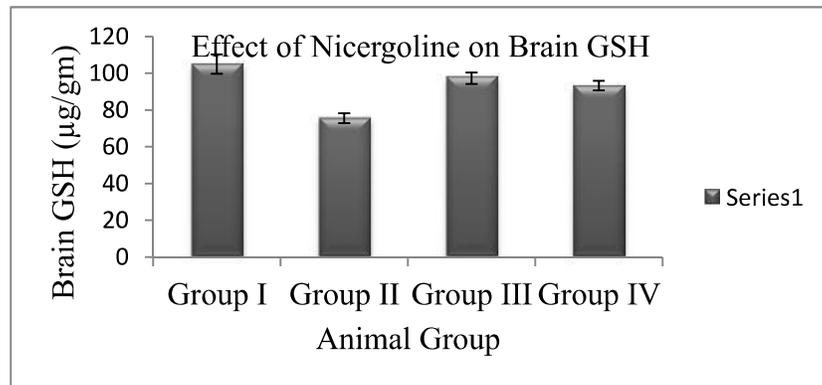**Figure 10.3** Effect of NG on brain edema

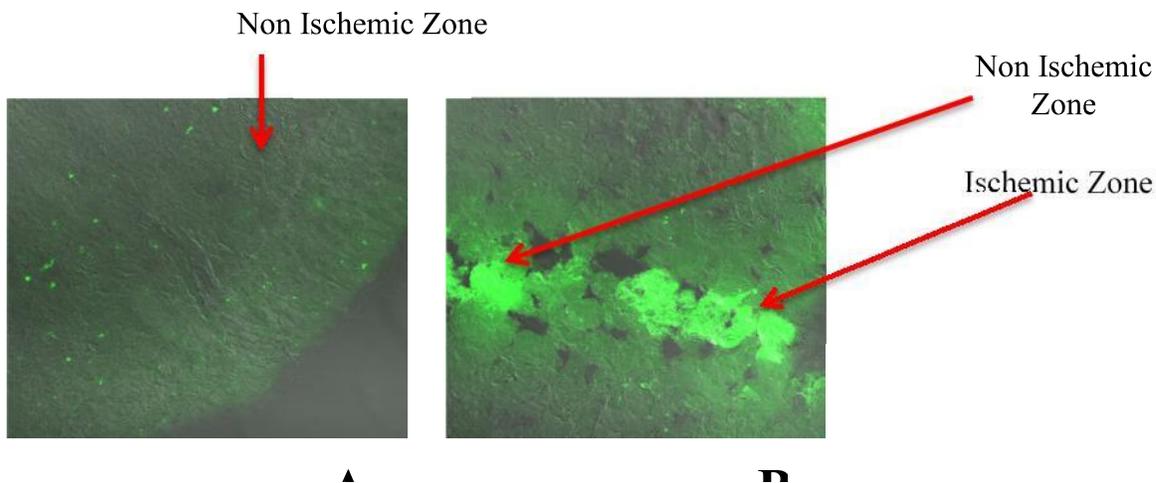**Figure 10.4** Effect of NG on brain glutathione (GSH)

Figure 10.5 A) Brain section of rat treated with MGM B) Brain section of rat treated with NGPC.

Figure 10.4 shows targeting efficiency of stroke homing peptide conjugated micelle. Ischemic zone is much concentrated with Coumarin and incase of non-conjugated micelle ischemic zone shows very few amount of Coumarin concentration.

10.5 Discussion

Intravenous delivery has limited utility in managing ischemic stroke due to the presence of BBB and restriction of blood supply to the brain due to thrombus or embolism limiting drug ingress effectively in to the brain illustrating intranasal route to be advantageous in such circumstances. Therefore, we developed intranasal delivery formulation of nicergoline mixed micelle system. The results of in vivo studies show that, significantly low brain GSH levels were observed following treatment with NGPC 30 min after induction of transient global ischemia compared to group I while the difference between group III and group IV insignificant. Also, the infarct volume and brain oedema were found to be significantly less for group III and group IV compaired to ischemic control group. These demonstrate the neuroprotective effect of micelle encapsulated nicergoline in cerebral ischemia with intranasal delivery enhancing their magnitude to many times by virtue of selective and effective delivery to the brain. The neuroprotectives effect of nicergoline is attributed to its multiple mechanisms of action viz. antioxidant activity, antiplatelet activity and increasing cellular ATP in brain. The targeting efficiency of peptide conjugated micelle was more in comparison to non-conjugated micelle as observed in Figure

10.5. As mentioned in earlier report stroke homing peptide efficiently target to the ischemic penumbra our results are similar to it.

10.6 Conclusion

The above results conclusively demonstrate intranasal drug delivery a rational strategy to manage debilitating brain disorder like cerebral ischemia and related obesity delivering drugs quickly and in higher concentrations to the target tissue. Also demonstrate stroke homing peptide conjugated micelle efficiently target the ischemic zone in the brain efficiently and in higher concentrations in comparison to non-targeted micelle after intranasal administration to the target tissue.

10.7 References

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