Chapter 8

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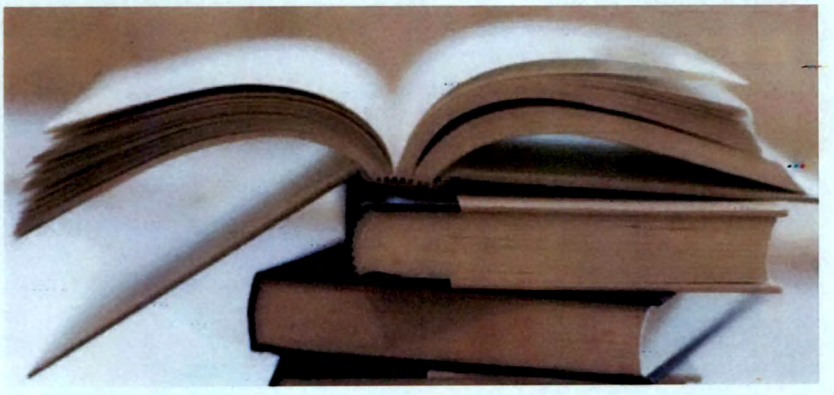

Summary

The treatment of CNS disorders are challenging because of a variety of formidable obstacles for effective and persistent delivery of drugs. Even though the drugs used for the treatment of CNS disorders are potent, their clinical failure is often not due to lack of drug efficacy but mainly due to short comings in the drug delivery approach. Intranasal drug delivery is one of the focused delivery option for brain targeting as brain and nose compartments are connected to each other via olfactory/ trigeminal route via peripheral circulation. Intranasal drug delivery delivers drug directly to the brain circumventing BBB and reduces drug delivery to the non targeted sites. This may result in reduction in dose, systemic dilution and first pass metabolism of the drug. Direct nose to brain transport results into rapid and/ of higher uptake in the brain, which provides an alternative option of self medication in the management of emergencies.

Despite enormous advances in brain research, brain and central nervous system disorders remain the world's leading cause of disability, and account for more hospitalizations and prolonged care than almost all other diseases combined. Patients suffering from fatal/and/or devastating CNS disorders such as neurogenerative disorders like alzheimer's disease, Parkinson;s disease, epilepsy, migraine, cerebrovascular diseases and HIV encephalopathy, far outnumber of those victimized from several types of systemic cancers and heart diseases. Beyond the loss of life, this broad category of disorders can have an overwhelming effect on the quality of life for the surviving patient and can lead to serious social and economic burdens on society. In developed nations, stroke is the third leading cause of death, only surpassed by heart disease and cancer. Despite the tremendous mortality and morbidity of stroke, treatment options remain limited. The most plausible reason for this failure is the multiplicity of mechanisms involved in causing neuronal damage during ischemia. Epilepsy is one of the most common of the serious neurological disorders. Beyond symptoms of the underlying diseases that can cause certain epilepsies, people with epilepsy are at risk for death from four main problems: status epilepticus (most often associated with anticonvulsant noncompliance), suicide associated with depression, trauma from seizures, and sudden unexpected death in epilepsy (SUDEP).

Clobazam *(7-Chloro-1,5-dihydro-1-methyl-5-phenyl-1,5-benzodiazepine- 2,4(3H)-dione)* is benzodiazepine derivative used in the treatment of CNS disorders like epilepsy,

anxiety and schizophrenia. Clobazam and its active metabolite, N-desmethylclobazam (norclobazam) are GABA agonists and longer half life (48hours and 72 hours). N-desmethyl clobazam is accumulated during long term treatment achieving concentration levels upto10 times greater than clobazam and therefore it may be an important factor in both therapeutic and toxic responses. It is mainly excreted in urine (87-91%) and accumulation is expected in impaired renal functions. The higher oral bioavailability and longer half life of clobazam can be additive in terms of tolerance, drug dependence and withdrawal symptoms associated with the long term use of benzodiazepines

Clopidogrel bisulphate (Thieno[3,2-c]pyridine-5(4H)-acetic acid, α-(2-chloro phenyl) 6,7-dihydro-,methyl ester,(S)-,sulfate (1:1)) is a thioenopyridine-derived antiplatelet drug which has been used for the secondary prevention of stroke. It inhibits ADP receptor/$P2Y_{12}$, thereby it inhibits ADP induced platelet aggregation which is important in stroke development, both in the pathogenesis of atherosclerosis and in the occurrence of acute cerebral artery occlusions. Inhibition of platelet aggregation occurs 24-48 h following the oral intake of clopidogrel and reaches its maximum level in 3 to 5 days. Restoration of platelet functions occurs slowly within 7-14 days, after withdrawal of the drug. Clopidogrel was shown to have neuroprotective effects on hippocampal regeion of brain against hypoxia.

Insulin like growth factor-1 is an investigation peptide drug and shown to effective in the protection of brain in ischemic events. Insulin-like growth factor-I (IGF-I) is a 70 amino acid neurotrophic factor with a molecular weight of 7649 Da and structural homology to proinsulin. The major source of IGF-I in the body is the liver, although it is expressed in many other tissues, including the CNS. Circulating IGF-I plays a prominent role in normal growth and development by mediating the indirect effects of growth hormone, with which it has a complex relationship. Despite of the high-affinity receptor for IGF-I is found on brain capillary endothelial cells, blood-borne IGF-I has difficulty in crossing the blood– brain barrier except in specific hypothalamic and anterior thalamic nuclei. IGF-I may be more efficiently transported across the blood–CSF barrier, but the IGF-I binding capacity present in both blood and CSF may hinder significant transport from the bloodstream into CNS parenchyma by this route. Previous studies showed that that intranasally delivered IGF-I from

solution can bypass the blood– brain barrier via olfactory and trigeminal-associated extracellular pathways to rapidly elicit biological effects at multiple sites within the brain and spinal cord.

Mucoadhesive based drug delivery systems gained importance because of longer residential time at the site of administration and possible enhanced transport mechanism by the mucoadhesive agents. Microemulsions were already explored as an effective carrier system for the rapid and larger uptake of the drugs like nimodipine, diazepam, sumatriptan and tacrine to the brain. Prolonged residence time of the formulations can be achieved by tailoring the viscosity of the formulation by the addition of various grades and types of mucoadhesive agents. Longer residence time is required for the absorption of high molecular weight compounds like peptides/ protein drugs. Nasal gels were found to be effective delivery system for peptide drugs like insulin and calcitonin.

The objectives of this investigation were to prepare and characterize micoemulsion/ mucoadhesive microemulsions of colbazam/ clopidogrel bisulphate and mucoadhesive gel of insulin like growth factor-1 and to assess their pharmacokinetic performance for brain drug delivery in mice after i.n. delivery. It was also an objective to assess their role pharmacodynamically in suitable animal models. It was hypothesized that i.n administration of clobazam/ clopidogrel bisulphate ME/ MME and nasal gel of IGF-1 will result in selective and effective nose-to-brain transport, reduce drug distribution in other parts of body and reduce side effects in the treatment of epilepsy and ischemic stroke.

8.1. Analytical methods

The UV spectroscopic method described in the USP was used for the clobazam estimation. Clobazam from excipients and formulations was estimated at 230nm. In the presence of interfereing excipients, derivatization method was used. The method was validated for linearity, accuracy and precision. The validation parameters were found to meet the "readily pass criteria" specified in the USP. The UV method was found to be linear in the range of 1-6 µg /ml with r^2 value of 0.9983. Estimation of clobazam in the diffusion samples was done by HPLC method. The elution was made with the flow rate of 1ml/min of acetonitrile : water (70:30) and the retention time of clobazam was found to be 5.18 minutes at 230nm of detection. The

linearity of the estimation was found to be 0.9991 in the range of 250 ng/ml - 50μg/ml. No interference was observed in the HPLC estimation, since the drug was extracted from excipients and the components of the diffusion media while elution.

A simple UV spectroscopic method was developed from the monographs of clopidogrel bisulphate from the British Pharmacopoeia. Clopidogrel bisulphate from excipients and formulations was estimated at 270nm. In the presence of interfereing excipients, derivatization method was used. The method was validated for linearity, accuracy and precision. The validation parameters were found to meet the "readily pass criteria" specified in the USP. The UV method was found to be linear in the range of 5-250 μg /ml with r^2 value of 0.999. Estimation of clopidogrel bisulphate in the diffusion samples was done by HPLC method. The elution was done with the flow rate of 1.2ml/min of Acetonitrile: Methanol: Sodium dihydrogen Phosphate buffer (pH 3) (70: 5: 25) and the retention time of clopidogrel bisulphate was found to be 8.84 minutes at 240nm of detection. The linearity of the estimation was found to be 0.9996 in the range of 1 - 50 μg/ml. No interference was observed in the HPLC estimation.

8.2. Preparation and Characterisation of formulations

Microemulsions of clobazam were successfully prepared using titration method followed by construction of pseudo ternary phase diagram. Based on the solubility study data, Capmul MCM was selected as an internal phase for the preparation of microemulsion. Surfactants, Tween 20 and Acconan CC6 were selected for the study along with cosurfactants like Transcutol P and PEG 200. For clobazam, 2 systems were prepared which are System1 [Capmul MCM, Acconan CC6: Tween 20(3:1), Distilled Water] and System2 [Capmul MCM, Tween 20: Transcutol P (3:1), Distilled Water]. Experimental design (3^2) was applied in the formulation of microemulsion by varying oil content from 2.5%v/v to 7.5%v/v and S_{mix} from 30%v/v to 50%v/v, measuring globule size (GS) and zeta potential (ZP) as the responses.

Microemulsions of clopidogrel bisulphate were successfully prepared using titration method followed by construction of pseudo ternary phase diagram. Based on the solubility study data, Capmul GMO was selected as an internal phase for the

preparation of microemulsion. Surfactant Tween 20 and Tween 80 were selected for the study along with cosurfactants like Transcutol P and PEG 200. For Clopidogrel bisulphate , three systems were prepared which are System1 [Capmul GMO, Tween 80:Transcutol P(2:1), Distilled water], System2 [Capmul GMO, Tween 20: Transcutol P(2:1), Acetate buffer (pH 5)] and System3 [Capmul GMO, Tween 20:PEG 200 (3:1), Acetate buffer (pH 5)] Experimental design (3^2) (Table 4.17 & 4.21) was applied in the formulation of microemulsion by varying oil content from 2.5%v/v to 7.5%v/v and S_{mix} from 40%v/v to 70%v/v, measuring globule size (GS) and zeta potential (ZP) as the responses.

The prepared microemulsions/ mucoadhesive microemuslions were characterized for globule size, zeta potential, transparency, % assay, viscosity, pH, refractive index and TEM. It was found that the addition of carbopol and chitosan to ME tends to increase the zetapotential in negative and positive side respectively. The globule size and zeta potential were fairly reproducible within ± 5nm / ± 5mv. The pHs of the formulations were found to be within the range of nasal cavity secretions. Microemulsions were found to possess lower viscosity and exhibit Newtonian flow. The formulations were studied for nasal toxicity and the optical microscopy images of nasal mucosa treated with CZ and CS formulations were taken. The sheep nasal mucosa treated with PBS pH 6.4 and isopropyl alcohol were taken as positive and negative control respectively. On observation, mucosas treated with formulations were found to be intact/ without much damage of the epithelial layer, the prepared formulations were found to be comparatively safe on nasal mucosa than isopropyl alcohol. In long term stability study, the CZME and CSME microemulsions were packed in the borosil screw capped vials and were kept at room temperature (25-35°C) and refrigeration temperature (20°C). During the storage period, microemulsion systems were assessed for their zeta potential, globule size, physical stability, assay and pH. Over the time period of 6 months, the slight increment in the zeta potential and globules size were found which was not significant when no visual indications of physical instability of microemulsion was seen. Irrespective of the storage conditions, the clobazam and clopidogrel bisulphate microemulsion systems remained stable for 6 months duration. In order to assess the thermodynamic stability, the accelerated stability studies were done by subjecting the formulations for centrifugation, freeze-

thaw cycle and heating cooling cycle. Before and after each treatment, zeta potential, globule size and %transmittance of the formulations were measured and were found to be insignificant which clearly indicates that the prepared microemulsion systems were thermodynamically stable.

8.3 *In vitro* diffusion study

The in-vitro diffusion studies for containing clobazam and clopidogrel bisulphate formulations were performed in 20% v/v methanolic phosphate buffer pH 5 containing 0.4% v/v Tween 80 and phosphate buffer pH 5 containing 0.2% v/v Tween 80 respectively. The % cumulative drug diffused across nasal mucosa from the formulations was calculated. The kinetic pattern of the diffusion was studied by fitting % drug diffused in given time in different order kinetics like zero order, first order and higuchi order. Regression coefficients of all formulations in different orders were compared and found that the release pattern of clobazam / clopidogrel from the formulation across the nasal mucosa followed higuchi order rather than zero order and first order. This was concluded by higher regression coefficient value in curve fitting. Among clobazam, clopidogrel bisulphate formulations, CZMME with highest flux (0.0775 µg/ min) and diffusion coefficient (1.03E-04 mm^2/ min) and CSMME with highest flux (0.0669 µg/ min) and diffusion coefficient (6.69E-05 mm^2/ min) respectively were selected for further pharmacokinetic study.

8.4. Pharmacokinetic study

Clobazam formulations (CZS, CZME and CZMME) were radiolabeled by direct labeling method. The radiolabeling was optimized by taking three factors in consideration, amount of stannous chloride, incubation time/ *in vitro* stability of the radiolabeled complex and pH. The amount of stannus chloride for optimum labeling of CZ formulations was found to be 200-250µgm in the pH range of 6- 8.5. The clobazam formulations were labeled successfully and their radio chemical purity/ labeling efficiency were found to be more than 90%. The radiolabeled ^{99m}Tc-CZS/CZME/CZMME complexes were found to be stable in normal saline and mice serum only with 10% degradation over 24 hours. The percent transchelation of ^{99m}Tc-CZS/CZME/CZMME were found to be less than 4% with 4mM DTPA.

Biodistribution of ^{99m}Tc-CZS following i.v. and ^{99m}Tc-CZS/CZME/CZMME following i.n. administration in Balb/c mice were performed and the radioactivity was estimated at predetermined time intervals up to 24hours. The pharmacokinetic parameters Cmax, Tmax, AUC, terminal elimination rate constant, half life and nasal bioavailability were calculated using standard pharmacokinetic principles. Lower T_{max} values (0.5 hour) were observed for i.n. CZME and CZMME. This may be attributed to preferential nose to brain transport following nasal administration. The brain/blood ratios of drug at all time points were found tobe higher following i.n. administration of the formulations than i.v solution. This further confirms direct nose to brain transport. The higher T_{max} and lower C_{max} of clobazam in brain following i.n. CZS can be better explained by the rapid nasal ciliary clearance of the solution from the site of administration. The higher concentration of clobazam in brain following i.n administration of CZME and CZMME demonstrates the suitability/ capability of microemulsion as an effective delivery system across the nasal membrane and a larger extent of selective transport of clobazam from nose to brain. CZMME was shown to have significantly higher C_{max} and AUC of CZMME demonstrated the importance of the mucoadhesive agent in prolonging the contact time of the formulation with the nasal mucosa and thereby enhancing rate and extent of absorption of drug. The nasal bioavailability of clobazam in brain following i.n. of ^{99m}Tc-CZS /CZME /CZMME was 51.68%, 158.93% and 196.32% respectively. The brain targeting efficiency of CZ formulations were calculated by the indices %DTE (Drug targeting efficiency) and %DTP (Direct nose-to-brain transport). Among all the three nasally administered formulations, CZMME showed highest %DTE (70.09%) and %DTP (91.25%) values followed by CZME (64.98% & 90.58%) and CZS (29.98% & 79.54%) respectively. These findings demonstrated the mucoadhesive microemulsion has higher brain targeting efficiency by the virtue of bioadhesion and lipophilicity of the delivery system.

In order to ascertain the brain uptake following i.v and i.n of the formulations, the gamma scintigraphy images of rabbits 30 min post i.v. and i.n. administrations were recorded. The presence of radioactivity in the esophagus and GIT may be due to possible ingestion of formulation by the animal while i.n. administration. Accumulation of radioactivity in the rabbit brain following different formulations and

route of administration was observed and found that intranasal CZMME showed significantly high radioactivity in the brain. The scintigraphy images were consistent with the findings of the biodistribution studies.

Clopidogrel bisulphate formulations (CSS, CSME and CSMME) were radiolabeled by direct labeling method with reduced technetium. The radiolabeling was optimized by taking three factors in consideration, amount of stannous chloride, incubation time/ *in-vitro* stability of the radiolabeled complex and pH. The amount of stannus chloride for optimum labeling of CS formulations was found to be 200μgm in the pH range of 6- 7. The clopidogrel bisulphate formulations were labeled successfully and their radio chemical purity/ labeling efficiency were found to be more than 92%. The radiolabeled ^{99m}Tc-CSS/CSME/CSMME complexes were found to be stable in normal saline and mice serum only with 10% degradation over 24 hours. The percent transchelation of ^{99m}Tc-CSS/CSME/CSMME were found to be less than 3% with 4mM DTPA.

Biodistribution of ^{99m}Tc-CSS following i.v. and ^{99m}Tc-CSS/CSME/CSMME following i.n. administration in Balb/c mice were performed and the radioactivity was estimated at predetermined time intervals up to 24hours. The pharmacokinetic parameters Cmax, Tmax, AUC, terminal elimination rate constant, half life and nasal bioavailability were calculated using standard pharmacokinetic principles. Lower T_{max} values (0.5 hour) were observed for i.n. CSME and CSMME. This may be attributed to preferential nose to brain transport following nasal administration. The brain/blood ratios of drug at all time points were found to be higher following i.n. administration of the formulations than i.v solution. The higher T_{max} and lower C_{max} of clopidogrel bisulphate in brain following i.n. CSS can be better explained by the rapid nasal ciliary clearance of the solution from the site of administration and dissociation of drug at nasal pH. The concentration of clopidogrel in brain at 0.5hr following i.n administration of CSME and CSMME were 0.204 and 0.239 which demonstrates the suitability/ capability of microemulsion as an effective delivery system across the nasal membrane and a larger extent of selective transport of drug from nose to brain. The AUC in brain followed by i.n. CSME and CSMME are 0.696 and 1.006 respectively the high vales indicates that microemulsion enhances the transport of drug across nasal mucosa. CSMME was shown to have significantly higher C_{max} and

AUC of CSMME demonstrated the importance of the mucoadhesive agent in prolonging the contact time of the formulation with the nasal mucosa and thereby enhancing rate and extent of absorption of drug. The nasal bioavailability of clopidogrel in brain following i.n. of ^{99m}Tc-CSS /CSME / CSMME was 47.75%, 117.77% and 170.31% respectively. The brain targeting efficiency of CS formulations were calculated by the indices %DTE (Drug targeting efficiency) and %DTP (Direct nose-to-brain transport). Among all the three nasally administered formulations, CSMME showed highest %DTE and %DTP values followed by CSME and CSS. These findings demonstrated the mucoadhesive microemulsion has higher brain targeting efficiency by the virtue of bioadhesion and lipophilicity of the delivery system.

In order to ascertain the brain uptake following i.v and i.n of the formulations, the gamma scintigraphy images of rabbits 30 min post i.v. and i.n. administrations were recorded. The presence of radioactivity in the esophagus and GIT may be due to possible ingestion of formulation by the animal while i.n. administration. Accumulation of radioactivity in the rabbit brain following different formulations and route of administration was observed and found that intranasal CSMME showed significantly high radioactivity in the brain. The scintigraphy images were consistent with the findings of the biodistribution studies.

Insulin like growth factor -1 was radiolabeled by direct labeling method. The radiolabeling was optimized by taking three factors in consideration, amount of stannous chloride, incubation time/ *in-vitro* stability of the radiolabeled complex and pH. The amount of stannous chloride for optimum labeling was 100µg at the pH of 6.2. Radiolabeled IGF-1 was incubated with the gel and the stability of IGF1 in solution and gel were studied. The formulations were labeled successfully and their radio chemical purity/ labeling efficiency were found to be more than 90%. The radiolabeled ^{99m}Tc- IGF1S/ IGF1G complexes were found to be stable in normal saline and mice serum only with 15% degradation over 24 hours. The percent transchelation of ^{99m}Tc- IGF1S/ IGF1G were found to be less than 2.5% with 4mM DTPA.

Biodistribution of ^{99m}Tc-IGF1S following i.v. and ^{99m}Tc- IGF1S/ IGF1G following i.n. administration in Balb/c mice were performed and the radioactivity was

estimated at predetermined time intervals up to 24hours. The pharmacokinetic parameters C_{max}, T_{max}, AUC, terminal elimination rate constant, half life and nasal bioavailability were calculated using standard pharmacokinetic principles.

The brain/blood ratios of drug at all time points were found tobe higher following i.n. administration of the formulations than i.v solution. This confirms direct nose to brain transport of IGF-1. The low bioavailability of IGF-I in brain following i.v. was due to the limitation of IGF-1 to pass through BBB. The lower C_{max} of IGF-1 solution can be better explained by the rapid nasal ciliary clearance of the solution from the site of administration. The higher concentration of IGF-1 following i.n administration of IGF1S / IGF1G demonstrates the effective delivery across the nasal membrane and a larger extent of selective transport of drug from nose to brain. This is in agreement with many scientists who believe in this unique connection between the nose and brain and drug transport to brain circumventing the BBB after i.n. administration. The AUC of IGF-1 in brain followed by i.n. of IGF1S and IGF1G are 0.235 and 0.351 respectively. The substantial increment in C_{max} and brain concentrations following IGF-1G demonstrated the importance of the mucoadhesive agent in the delivery system. The mucoadhesive polymer carbopol and HPMC were shown to prolong the contact time of the formulation with the nasal mucosa and thereby enhancing rate and extent of absorption of drug. The nasal bioavailability of IGF-1 in brain following i.n. of ^{99m}Tc- IGF1S / IGF1G were 114.38% and 170.75% respectively. The brain targeting efficiency of IGF-1 formulations were calculated by the indices %DTE (Drug targeting efficiency) and %DTP (Direct nose-to-brain transport). Among the solution and nasal gel, IGF-1 Gel showed higher %DTE and %DTP values than IGF-1 solution. These findings demonstrated the mucoadhesive nasal gels are suitable delivery system for high molecular weight candidate drugs like peptides.

Gamma scintigraphy imaging of rabbits administered with i.v of ^{99m}Tc-IGF-1S and i.n.of ^{99m}Tc- IGF-1S / IGF-1G were performed in order to confirm drug localization in brain. The presence of radioactivity in the esophagus and GIT may be due to possible ingestion of formulation by the animal while i.n. administration. Accumulation of radioactivity in the rabbit brain following different formulations and route of administration was observed and found that IGF-1G showed significantly

high radioactivity in the brain. The scintigraphy images were consistent with the findings of the biodistribution studies.

8.5 Pharmacodynamic study

The anticonvulsant activity of clobazam formulations following intravenous and intranasal administration in PTZ induced convulsions in mice was studied and the delay in the onset of seizures was taken as the index for the animal's protection against convulsations. The prolonged delay in the onset of seizures in the $CZMME_{IN}$ treated groups ($p<0.05$) suggests that rapid and effective delivery of the drug to the brain. All significant groups (CZS_{IV} -30^{th} and 45^{th} groups, CZS_{IN} -30^{th}, $CZME_{IN}$ -30^{th} and 45^{th} groups and $CZMME_{IN}$ – 15^{th}, 30^{th} and 45^{th} groups) were compared among them and with control. The protection offered by $CZMME_{IN}$-15^{th} group was found to be rapid and effective delivery ($p< 0.05$). Thus the studies under investigation suggest that the rapid and effective protection against convulsion was achieved by the use mucoadhesive microemulsion by intranasal administration and it further supports the findings of the biodistribution studies.

The neuroprotective role of clopidogrel bisulphate as an antioxidant in transient global ischemia was studied and the level of glutathione in brain and blood were measured. The reduction in the GSH level in the ischemic group clearly indicates that the reservoir of antioxidant system get depleted during the ischemic events. The elevated level of brain GSH in treatment groups (CSS_{IN}, $CSME_{IN}$ and $CSMME_{IN}$) compared to control and CSS_{IV} indicate that the antioxidant system was restored due to the pretreatment of animals with clopidogrel bisulphate and the effective delivery of clopidogrel to the brain. The GSH level in the blood of CSS_{IV}, CSS_{IN}, $CSME_{IN}$ and $CSMME_{IN}$ treated groups and control group were found to be insignificant ($p< 0.05$) indicated that the GSH levels were restored during ischemic events. The significant ($p< 0.05$) rise in brain GSH in $CSMME_{IN}$ group indicate the effective delivery of drug to the brain. The studies under this investigation support the biodistribution studies.

The neuroprotective role of IGF-1 as an antioxidant in transient global ischemia was studied and the levels of total IGF-1 and glutathione in brain and blood were measured. High IGF-1 level was observed in IGF-1G treated group as compared to other treatment groups. The total circulating IGF-1 level in plasma in all treatment

groups were found to be increased due to ischemic event. There was no significant difference was found in the glutathione levels of control, IGF-$1S_{IV}$ and IGF-$1S_{IN}$ groups ($p< 0.05$) indicated that the antioxidant enzyme systems were restored in the IGF-1 treated animal groups. The elevated levels of IGF-1 and restoration of GSH levels in the IGF-1_{IN} treated groups suggest that intranasal administration of IGF-1 offers more neuroprotection against ischemia. The rise in the brain GSH level in IGF-1G group can be explained by the effective delivery by nasal gel and consequent IGF-1 signaling during ischemic events. The studies under this investigation indicate that the intranasal administration of mucoadhesive gel of IGF-1 is the effective delivery approach to the brain and further it supports the biodistribution studies.