Chapter 7

Pharmacodynamic Study

7.1. CLOBAZAM

7.1.1 Pentylene tetrazole (Metrazole) induced convulsions in mice:

All experiments conducted on animals were approved by the Committee for the purpose of control and supervision of experiments on animals, Ministry of Social Justice and Empowerment, Government of India, NewDelhi, India. Balb/c mice (aged 4 to 5 months) weighing between 20- 30 g were selected for study on the basis of randomization technique.

Epilepsy is a common chronic neurological disorder characterized by recurrent unprovoked seizures (CEP 1993, Blume 2001). These seizures are transient signs and/or symptoms of abnormal, excessive or synchronous neuronal activity in the brain (Fischer et al 2005). Clobazam and its active metabolite, N-desmethyl clobazam (norclobazam) work by enhancing GABA-activated chloride currents at $GABA_A$-receptor-coupled Cl^- channels (Nakamura et al 1996).

Pentylenetetrazole (PTZ) is a GABA antagonist and produces a reliable discriminative stimulus which is largely mediated by the $GABA_A$ receptor. Electrophysiological studies have shown that it acts at cell membrane level decreasing the recovery time between action potentials by increasing potassium permeability of the axon. Since PTZ has the epileptogenic action, it has been used experimentally to study seizure phenomenon and to identify pharmaceuticals that may control seizure susceptibility. It produces generalized asynchronized clonic movements which are superceded by tonic convulsions characterised by flexion of limbs followed by extension.

Materials and requirements:

Pentylene tetrazole was purchased from Sigma chemicals.

1 ml tuberculin syringe, 100µl micropipette

Animals:

Mice were used for the anticonvulsant study. Swiss albino mice of either sex (20-30 g) were used which were provided by Food and Drug Laboratory, Vadodara. The mice were housed as 6-8 per cage. The bedding was raised regularly otherwise the animals are expected to eat whatever they find (coprophagy) and even tail or skin of other animals.

Methodology:

The animals were made in groups each group consisted of 5 animals. In the control group, convulsions were induced by injecting the animal with 60mg/kg of pentylene

tetrazole. The average onset of seizures in mice was recorded. Four groups were made for each formulation of CZS_{IV}, CZS_{IN}, $CZME_{IN}$ and $CZMME_{IN}$. With in the formulation/ route of administration group, the time gap between the drug treatment and injection of pentylene tetrazole was varied as 15, 30 and 45 minutes. The fourth group was treated with the placebo formulation in order to study the effect of formulation excipients on the PTZ model. The animals were treated with the drug formulation with the dose of 0.75mg/ Kg of the body weight (Stuart Fielding et al 1982). For nasal administration of the formulations, mice were held from the back in slanted position and the formulations were instilled into the nostrils with the help of micropipette (10μL) attached with low density polyethylene tube having internal diameter at the delivery site. After 15, 30 and 45 minutes of formulation treatments, the mice were injected with pentylene tetrazole in normal saline by intraperitonial injection. Each animal was placed into an individual cage and observed for 15 – 30 minutes. The time interval between i.p. injection of PTZ and onset of seizures were recorded and represented in Fig. 7.1.

Statistical Analysis:

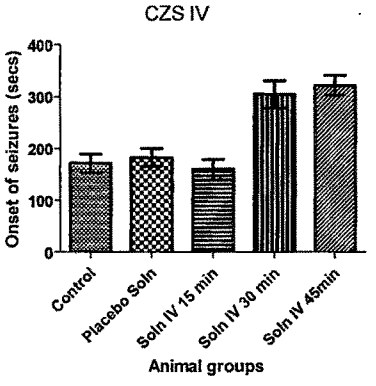
All data are reported as mean ± SD and all groups were compared using ANOVA followed by Dunnet test and differences greater at $P< 0.05$ were considered significant.

7.1.2 RESULTS AND DISCUSSION:

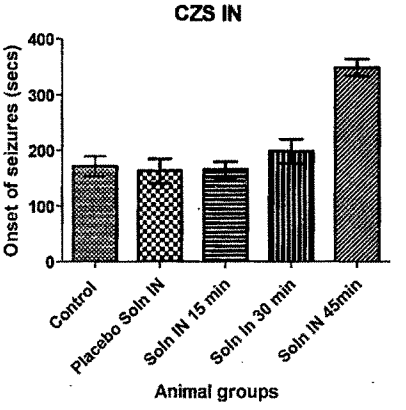
The anticonvulsant activity of clobazam formulations after intravenous and intranasal administration in PTZ induced convulsions in mice was studied and the delay in the onset of seizures was taken as the index for the animal's protection against convulsions. The onset of seizures in the treated animals were recorded and shown in Fig.7.1. It was observed that administration of clobazam solution by IV was able to protect the 30^{th} minute and 45th minute time group only (304.6 and 322.2 seconds)

Fig. 7.1 Effects of different clobazam formulations after IV and IN administration on onset of seizures induced by PTZ in mice (n=5)

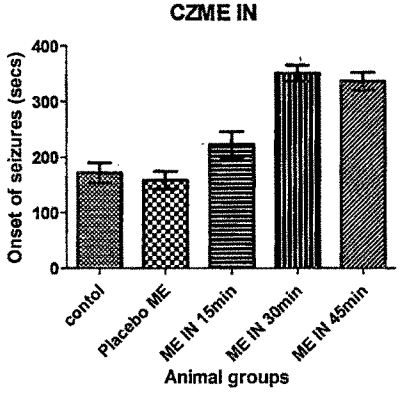
A. CZS IV treated



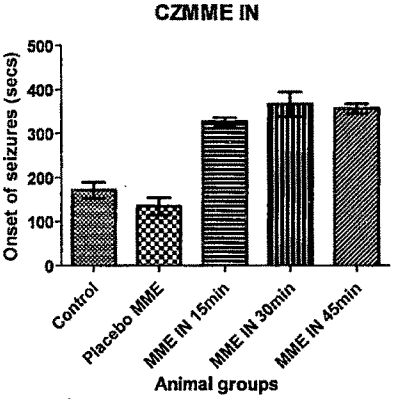
B. CZS IN treated



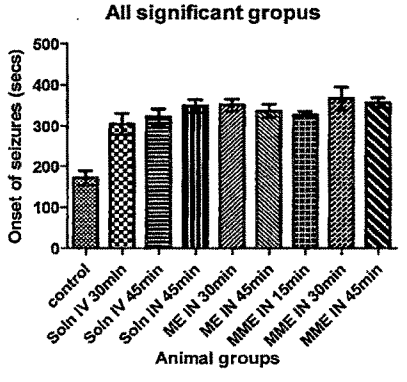
C. CZME IN treated



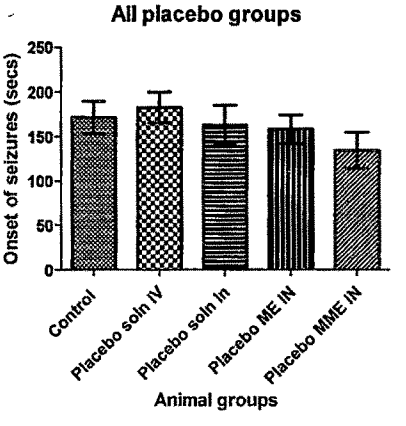
D. CZMME IN treated



E. All Significant groups



F. All placebo groups



In case of CZS_{IN}, only the 45th minute was protected (348.8 secs. Fig7.1B). This indicates the delay in the drug delivery to the brain which may be due to the lack of efficient delivery to the site of action. The protection of 30th minute group of $CZME_{IN}$ (Fig 7.1C) indicated that microemulsions are suitable carriers for the intranasal administration. The prolonged delay in the onset of seizures in the $CZMME_{IN}$ treated groups ($p<0.05$) (Fig 7.1D) suggests that there is rapid and effective delivery of the drug to the brain. All significant groups (CZS_{IV} -30th and 45th groups, CZS_{IN} -30th, $CZME_{IN}$ -30th and 45th groups and $CZMME_{IN}$ – 15th, 30th and 45th groups) were compared among them and with control and the protection offered by $CZMME_{IN}$-15th 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 can be achieved by the use of mucoadhesive microemulsion by intranasal administration and it further supports the findings of the biodistribution studies.

7.2 INSULIN LIKE GROWTH FACTOR-1

7.2.1 Transient global ischemia by carotid artery ligation

All experiments conducted on animals were approved by the Committee for the purpose of control and supervision of experiments on animals, Ministry of Social Justice and Empowerment, Government of India, NewDelhi, India. Balb/c mice (aged 4 to 5 months) weighing between 20- 30 g were selected for the study on the basis of randomization technique.

Insulin like growth factors (IGFs) are polypeptides with high sequence similar to insulin. This complex system/IGF axis consists of two cell surface receptors (IGF 1Rand IGF 2R), two ligands (IGF I and IGF II), a family of six high affinity IGF binding proteins (IGF BP 1-6), as well as associated IGFBP degrading enzymes, referred to collectively as proteases.

IGF treatment protects the developing or adult brain from hypoxic-ischemic injury (Gluckman et al 1992; Johnston et al 1996; Guan et al 1993 and 1996) and forebrain ischemia (Zhu et al 1994), induces myelination (McMorris et al 1993; Ye et al 1995 and Roth et al 1995) and reduces neuronal death *in vitro* caused by diverse forms of injury (Tagami et al 1997; Cheng et al 1992; Galli et al 1995; Sortino et al 1996). Several animal models have been developed in rats, mice and gerbils for the study of ischemic, hypoxic and hypoxic –ischemic brain injury. (Hossmann 1998; Vannucci et

al 1999) Global cerebral ischemia in mice was induced by two vessel occlusion model. Ligation of bilateral common carotid arteries by silk thread/ occlusion of both the arteries by aneurysm clips induce delayed neuronal death in animals (Durukan et al 2007; Candelario-Jalil 2003; Hwang 2006; Gillingwater 2004). GSH is key factor involved in the detoxification of electrophilic metabolites and reactive oxygen intermediates (Ferrari et al. 1986) and as a co-substrate of glutathione peroxidase, it plays an essential role in protection from free radicals (Curel o et al. 1985). Hence glutathione level in the brain and plasma was taken as the index for the anti oxidant activity of the IGF-1 (García-Fernández et al 2005; Donahue et al 2006). Additionally the IGF-1 levels in brain and plasma of the IGF-1 treated mice were also measured.

Material:

Insulin like growth factor-I was purchased from Sigma chemicals. Tris buffer and Disodium EDTA were purchased from Himedia laboratories limited, Mumbai. India. IGF-1was dissolved in phosphate buffer saline (PBS pH6).

Requirements:

Cold Tris buffer HCl (10mM pH 7.4), 2 % w/v Disodium EDTA solution, .Surgical equipments such as scissors, forceps, aneurysm clips etc. and instruments like Sigma 3K30 centrifuge, Deep freezer

Animals:

The mice were used for this hypoxic induction. Swiss albino mice of either sex (20-30 g) were used which were provided by Sun Pharma Advanced Research Company Ltd (SPARC), Vadodara. The mice were housed as 6-8 per cage. The bedding was raised regularly otherwise the animals are expected to eat whatever they find (coprophagy) and even tail or skin of other animals.

Methodology:

Swiss albino mice of either sex (20-30 g) were used. The animals were made in groups and each group consisted of 3 animals. Five groups were made and treated with IGF-1 solution by IV injection, IGF-1 solution by IN, IGF-1 Gel by IN, control group/sham operated group and ischemic group. The animals were treated with the dose of 20µg IGF-1/ Kg BW/ day for 5 days to mice intravenously and intranasally. The animals were anaesthetized with diazepam (5mg/Kg BW) and ketamine (44mg/Kg BW). 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 both common carotid arteries were surgically exposed. Taking care not to damage the vagus nerve, the common arteries were isolated using silk thread. After stabilization for 5 minutes, both the arteries were occluded with aneurysm clips for 30 minutes. Subsequently, the clips were removed and restoration of blood flow was confirmed before the incision was sutured closed. After 30 minutes of ischemia, reperfusion was allowed for 4 hours. Then animals were anesthetized with ketamine (100mg/kg), blood was collected by cardiac puncture method and collected in EDTA solution containing tubes. Brain was removed and washed with cold saline. Brain was then blotted, weighed and suspended in 0.25M cold sucrose solution and again blotted on a filter paper. The tissue was minced, homogenized with 10 volumes of cold Tris buffer HCl pH 7.4 and centrifuged at 10,000rpm for 20 minutes at 4°C. The clear supernatant was used for the biochemical analysis. The tissue homogenate and plasma samples were stored at -40°C until used for analysis.

Estimation of IGF-1:

The estimation of IGF-1 (both bound and free) was done with Novum International ELISA kit. This assay employs an antibody specific for human IGF-BP-1 coated on a 96-well plate. Standards and samples were pipetted into the wells and IGF-BP-1 present in a sample was bound to the wells by the immobilized antibody. The wells were washed and biotinylated anti-human IGF-BP-1 antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted into the wells. The wells were again washed, a 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to the wells and color develops in proportion to the amount of IGF-BP-1 bound. The reaction stop solution changes the color from blue to yellow, and the intensity of the color was measured at 450 nm.

Estimation of Glutathione:

Glutathione in the samples were measured with kinetic method based kit from Crest Biosystems, India. The biological sample was first deproteinized with the 5% 5-Sulfosalicylic acid solution, centrifuged to remove the precipitated protein, and then assayed for glutathione The measurement of GSH uses a kinetic assay in which catalytic amounts of GSH cause a continuous reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to TNB and the GSSG formed is recycled by glutathione reductase and NADPH. The GSSG present will also react to give a positive value in this reaction.

1. $2\,GSH + DTNB \longrightarrow GSSG + 2\,TNB$

2. $GSSG + NADPH + H^{+} \xrightarrow[\text{reductase}]{\text{Glutathione}} 2\,GSH + NADP^{+}$

The combined reaction:

$$DTNB + H^{+} + NADPH \xrightarrow[GSSG/GSH]{\text{Glutathione reductase}} 2\,TNB + NADP^{+}$$

The reaction rate is proportional to the concentration of glutathione up to 2 μM. The yellow product, 5-thio-2-nitro benzoic acid (TNB) was measured spectrophotometrically at 412 nm.

Statistical Analysis:

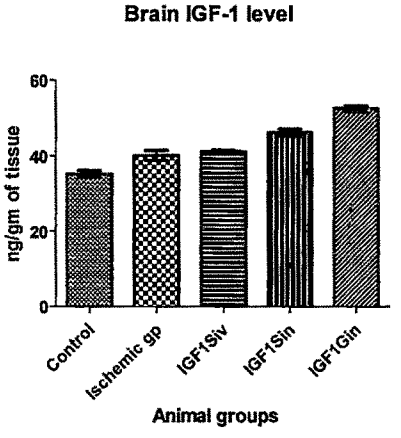
All data are reported as mean ± SD and all groups were compared using ANOVA followed by Dunnet test and differences greater at $P < 0.05$ were considered significant.

7.2.2 RESULTS AND DISCUSSION:

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 and shown in Fig 7.2 and Fig 7.3. The treatment of mice with IGF-1

Fig 7.2 Brain and Plasma levels of IGF-1 in IGF-1 treated mice (n=3)

A. Brain IGF-1 levels



B. Plasma IGF-1 levels

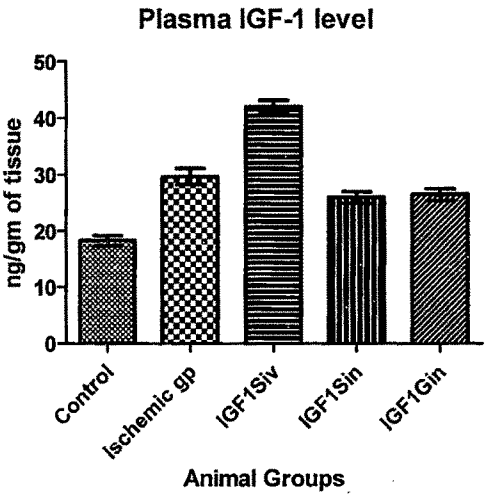
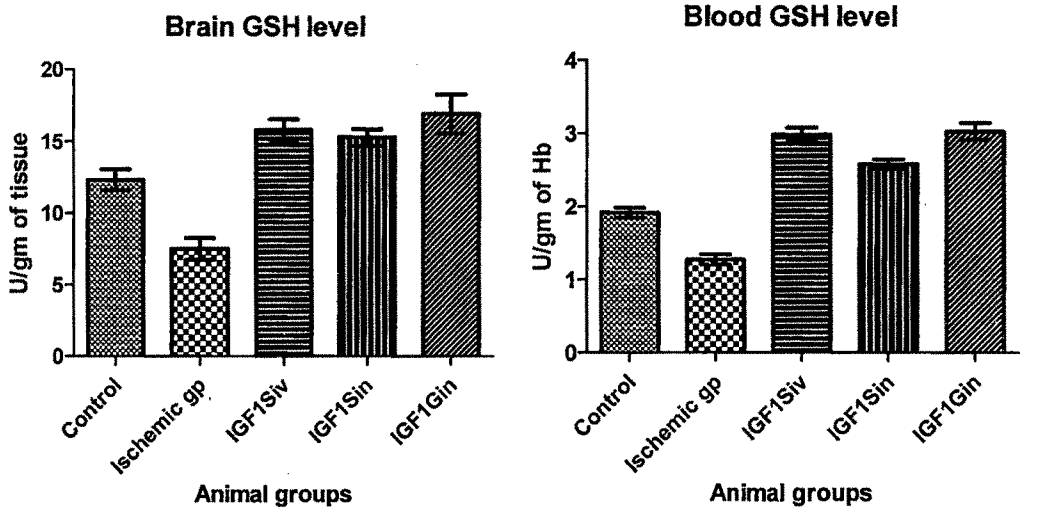


Fig 7.3 Brain and Plasma levels of Glutathione in IGF-1 treated mice (n=3)

A. Brain glutathione levels **B. Blood glutathione levels**



formulations showed significant rise in the brain IGF-1 level when compared to control group. The rise in the IGF-1 level after ischemic events was explained by Gluckman et al 1992. Thus the treatment with IGF-1 formulations and experimental cerebral ischemia raised the brain IGF-1 levels in all the groups (Fig 7.2 A). In case of plasma IGF-1, the total circulating IGF-1 level in plasma in treated groups were also found to be increased by the ischemic events (Fig 7.2 B).

The GSH levels in the brain of all groups were shown in Fig 7.3A. 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.

7.3 CLOPIDOGREL BISULPHATE

7.3.1 Transient global ischemia by carotid artery ligation

Huber et al 2005 demonstrated that clopidogrel bisulphate has neuroprotective property against the hypoxic injury in rodents. Clopidogrel is shown to be beneficial in prevention of ischemia reperfusion injury probably via its effects on inflammatory cells, platelets and endothelial cells. Morale H. et al 2005 studied the antioxidant role of clopidogrel by measuring oxidative stress markers like malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase (SOD) activity in tissue and plasma following ischemia reperfusion injury. Several animal models have been developed in rats, mice and gerbils for the study of ischemic, hypoxic and hypoxic –ischemic brain injury. (Hossmann KA. 1998; Vannucci et al 1999) Global cerebral ischemia in mice was induced by two vessel occlusion model. Ligation of bilateral common carotid arteries by silk thread/ occlusion of both the arteries by aneurysm clips induce delayed neuronal death in animals (Durukan et al 2007; Candelario-Jalil 2003; Hwang 2006; Gillingwater 2004). Since GSH is key factor involved in the detoxification of electrophilic metabolites and reactive oxygen intermediates (Ferrari et al. 1986) and as a co-substrate of glutathione peroxidase, it plays an essential role in protection from free radicals (Curello et al. 1985). Hence glutathione level in the brain and plasma was taken as the index for the anti oxidant activity of clopidogrel bisulphate.

Material:

Tris buffer and Disodium EDTA were purchased from Himedia laboratories limited, Mumbai. India.

Requirements:

Cold Tris buffer HCl (10mM pH 7.4), 2 % w/v Disodium EDTA solution., Surgical equipments such as scissors, forceps, aneurysm clips etc. and instruments like Sigma 3K30 centrifuge, Deep freezer

Animals:

The mice were used for this hypoxic induction. Swiss albino mice of either sex (20-30 g) were used which were provided by Sun Pharma Advanced Research Company Ltd (SPARC), Vadodara. The mice were housed as 6-8 per cage. The bedding was raised regularly otherwise the animals are expected to eat whatever they find (coprophagy) and even tail or skin of other animals.

Methodology:

Swiss albino mice of either sex (20-30 g) were used. The animals were made in groups and each group consisted of 5 animals. Six groups were made and treated with CSS by IV, CSS by IN, CSME by IN, CSMME by IN, control group and ischemic group. Sham operated /control group animals were treated with saline. The animals were pretreated with clopidogrel formulations (1mg/kg of BW/day for 10 days) to mice intravenously and intranasally. The animals were anaesthetized with diazepam (5mg/Kg BW) and ketamine (44mg/Kg BW). 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 both common carotid arteries were surgically exposed. Taking care not to damage the vagus nerve, the common arteries were isolated using silk thread. After stabilization of 5 minutes, both the arteries were occluded with aneurysm clips for 30 minutes. Subsequently, the clips were removed and restoration of blood flow was confirmed before the incision was sutured closed. After 30 minutes of ischemia, reperfusion was allowed for 4 hours. Then animals were anaesthetized with ketamine (100mg/kg), blood was collected by cardiac puncture method and collected in EDTA solution containing tubes. Brain was removed and washed with cold saline. Brain was then blotted, weighed and suspended in 0.25M cold sucrose solution and again blotted on a filter paper. The tissue was minced, homogenized with 10 volumes of cold Tris buffer HCl pH 7.4 and centrifuged at 10,000rpm for 20 minutes at 4°C. The clear supernatant was used for the biochemical analysis. The tissue homogenate and plasma samples were stored at -40°C until used for analysis.

Estimation of Glutathione:

Glutathione in the samples were measured with kinetic method based kit from Crest Biosystems, India. The biological sample was first deproteinized with the 5% 5-Sulfosalicylic Acid Solution, centrifuged to remove the precipitated protein, and then assayed for glutathione The measurement of GSH uses a kinetic assay in which catalytic amounts of GSH cause a continuous reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to TNB and the GSSG formed was recycled by glutathione reductase and NADPH. The GSSG present will also react to give a positive value in this reaction.

1. $2\,GSH + DTNB \longrightarrow GSSG + 2\,TNB$

2. $GSSG + NADPH + H^+ \xrightarrow[\text{reductase}]{\text{Glutathione}} 2\,GSH + NADP^+$

The combined reaction:

$$DTNB + H^+ + NADPH \xrightarrow[GSSG/GSH]{\text{Glutathione reductase}} 2\,TNB + NADP^+$$

The reaction rate is proportional to the concentration of glutathione up to 2 μM. The yellow product, 5-thio-2-nitro benzoic acid (TNB) was measured spectrophotometrically at 412 nm.

Statistical Analysis:

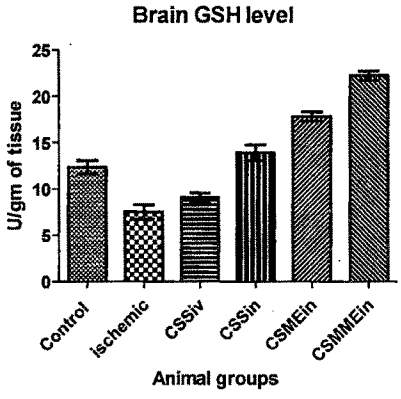
All data are reported as mean ± SD and all groups were compared using ANOVA followed by Dunnet test and differences greater at $P< 0.05$ were considered significant.

7.3.2 RESULTS AND DISCUSSION:

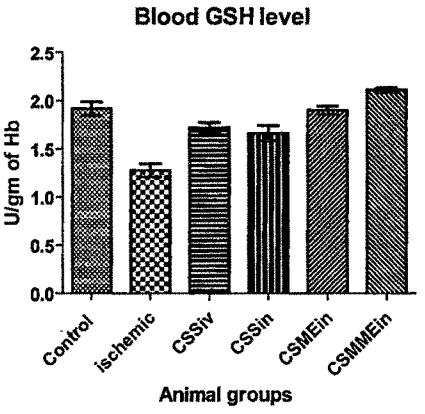
The neuroprotective role of clopidogrel bisulphate as an antioxidant in transient global ischemia was studied and the level of glutathione in brain and blood were measured and shown in Fig 7.4. The reduction in the GSH level in the ischemic group clearly indicates that the reservoir of antioxidant system get depleted during the ischemic events. The elevated level of 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 insignificant variation in the brain GSH levels of CSS_{IN}, $CSME_{IN}$ and $CSMME_{IN}$ treated groups with control group ($p< 0.05$) indicated the possible activation of clopidogrel in the nasal mucosa and protection of animals against ischemia. 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.

Fig 7.4 Brain and Plasma levels of Glutathione in Clopidogrel bisulphate treated mice (n=5)

A. Brain glutathione levels



B. Blood glutathione levels



7.4 REFERENCES

1. Blume W, Lüders H, Mizrahi E, Tassinari C, van Emde Boas W, Engel J. "Glossary of descriptive terminology for ictal semiology: report of the ILAE task force on classification and terminology". *Epilepsia* 2001; 42 (9): 1212–8.
2. Candelario-Jalil E; Dalia Alvarez; Nelson Merino and Olga Sonia León Delayed treatment with nimesulide reduces measures of oxidative stress following global ischemic brain injury in gerbils. *Neuroscience Research* 2003; 47(2): 245-253.
3. Cheng B. and Mattson MP. IGF-I and IGF-II protect cultured hippocampal and septal neurons against calcium-mediated hypoglycemic damage. *J. Neurosci.1992;* 12: 1558–1566.
4. Commission on Epidemiology and Prognosis, (CEP) International League against Epilepsy. "Guidelines for epidemiologic studies on epilepsy. Commission on Epidemiology and Prognosis, International League against Epilepsy". *Epilepsia* 1993; 34 (4): 592–6.
5. Curello S, Ceconi C, Bigoli C, Ferrari R, Albertini A. and Guarnieri C. Changes in the cardiac glutathione status after ischemia and reperfusion. *Experientia 1985;* 15: 42-43.
6. Donahue AN, Aschner M, Lash LH, Syversen T, Sonntag WE. Growth hormone administration to aged animals reduces disulfide glutathione levels in hippocampus. *Mech Ageing Dev.* 2006; 127(1):57-63.
7. Durukan A, Turgut Tatlisumak Acute ischemic stroke: Overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia.*Pharmacology Biochemistry and Behavior* 2007; 87(1) :179-197
8. Ferrari R, Ceconi C, Curello S, Cargnoni A, Albertini A and Visioli O. Oxygen utilization and toxicity at myocardial level. In: *Bio-chemical Aspects of Physical Exercise*, edited by G. Benzi, L. Packer & N. Siliprandi, Elsevier, Amsterdam, 1986:145-156.
9. Fielding S, Lal H. A review of the animal pharmacology of clobazam, an update. *Drug Development Research Supplement* 1982; 1: 17 -21.
10. Fisher R, van Emde Boas W, Blume W, Elger C, Genton P, Lee P and Engel J. "Epileptic seizures and epilepsy: definitions proposed by the International

League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE)". *Epilepsia* 2005; 46 (4): 470–2.

11. Galli C, Meucci O, Scorziello A, Werge TM, Calissano P and Schettini G. Apoptosis in cerebellar granule cells is blocked by high KCl, forskolin, and IGF-1 through distinct mechanisms of action: the involvement of intracellular calcium and RNA synthesis. *J. Neurosci.* 1995;15: 1172–1179.
12. García-Fernández M, Castilla-Cortázar I, Díaz-Sanchez M, Iñigo Navarro, Juan Enrique Puche, Alberto Castilla, Amelia Díaz Casares, Encarna Clavijo, and Salvador González-Barón. Antioxidant effects of insulin-like growth factor-I (IGF-I) in rats with advanced liver cirrhosis_*BMC Gastroenterol.* 2005; 5: 7.
13. García-Fernández, Inma Castilla-Cortázar, Matías Díaz-Sanchez, Iñigo Navarro, Juan Enrique Puche, Alberto Castilla, Amelia Díaz Casares, Encarna Clavijo, and Salvador González-Barón. Antioxidant effects of insulin-like growth factor-I (IGF-I) in rats with advanced liver cirrhosis_*BMC Gastroenterol.* 2005; 5: 7.
14. Gillingwater TH, Haley JE, Ribchester RR, Horsburgh K. Neuroprotection after transient global cerebral ischemia in mutant mice. *J Cereb Blood Flow Metab.*2004; 24(1):62-66.
15. Gluckman P, Klempt N,Guan J, Mallard C, Sirimanne E, Dragunow m et al. a role for IGF1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochem Biophys Res Commun* 1992; 182: 593-59.
16. Hossmann KA Experimental models for the investigation of brain ischemia. *Cardiovas Res* 1998;39:106-120
17. Huber R, Reepe MW. Improved posthypoxic recovery invitro on treatment with drugs used for secondary stroke prevention. *Neuropharmacology* 2005;48: 558-565
18. Hwang IK, Ki-Yeon Yoo, Duk Soo Kim Protective effects of grape seed proanthocyanidins on cerebral ischemia-reperfusion injury in mice. *Chinese Journal of Clinical Rehabilitation* March 14, 2006.
19. Johnston BM, Mallard EC, Williams CE & Gluckman PD. Insulin-like growth factor-1 is a potent neuronal rescue agent following hypoxic-ischemic injury in fetal lambs. *J Clin Invest* 1996; 97: 300–308.

20. McMorris FA, Mozell, R L, Carson M J, Shinar Y, Meyer RD & Marchetti N. Regulation of oligoden-drocyte development and central nervous system myelination by insulin – like growth factors. *Ann. N. Y. Acad. Sci.* 1993; 692, 321–334.

21. Morale H, Knako M, Akbas MH, Ozden M et al. Protective effects of clopidogrel on oxidant damage in a rat model of acute ischemia. *Tohoku J Exp Med* 2005;205:133-139

22. Nakamura, Fumihiro; Suzuki Setsuo, Nishimura Shigeko, Yagi Kazuichi, and Seino Masakazu (1996). "Effects of clobazam and its active metabolite on GABA-activated currents in rat cerebral neurons in culture". *Epilepsia* 37 (8): 728–35.

23. Roth GA, Spada V, Hamill K, *et al.* Insulin-like growth factor I increases myelination and inhibits demyelination in cultured organotypic nerve tissue. *Brain Res Dev Brain Res* 1995;88:102–108.

24. Roth GA, Spada V, Hamill K, *et al.* Insulin-like growth factor I increases myelination and inhibits demyelination in cultured organotypic nerve tissue. *Brain Res Dev Brain Res* 1995;88:102–108.

25. Sortino MA and Canonico PL. Neuroprotective effect of insulin-like growth factor I in immortalized hypothalamic cells .*Endocrinology* 1996; 137:1418–1422.

26. Tagami M, Yamagata K, Nara Y, Fujino H, Kubota A, Numano F, Yamori Y. Insulin-like growth factors prevent apoptosis in cortical neurons isolated from stroke-prone spontaneously hypertensive rats. *Lab Invest* 1997; 76:603– 612.

27. Vannucci RC, Connor JR, Mauger DT, et al. Rat model of perinatal hypoxic – ischemic brain damage. *J NeuroSci Res* 1999;55:158-163)

28. Ye P, Carson J, D'Ercole AJ *In vivo* actions of insulin-like growth factor-1 (IGF-1) on brain myelination: studies of IGF-1 and IGF binding protein-1 (IGFBP-1) transgenic mice. *J Neurosci* 1995;15: 7344-7356.

29. Zhu CZ, Auer RN. Intraventricular administration of insulin and IGF-1 in transient forebrain ischemia. *J Cereb Blood Flow Metab* 1994; 14:237–242.