IN VITRO MODEL OF CEREBRAL STROKE: THERAPEUTIC POTENTIAL OF *TRANS* RESVERATROL AND CURCUMIN

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

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JANUARY, 2010

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE, ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "***IN VITRO* MODEL OF CEREBRAL STROKE:THERAPEUTIC POTENTIAL OF *TRANS* RESVERATROL AND CURCUMIN"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from July 2005 to January 2010 under the supervision of Dr. G.S. Randhawa, Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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G. S. Randhawa 19.10.2010

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ABSTRACT

Ischemic cerebral stroke, which occurs due to loss of blood supply to the brain, is the second largest cause of morbidity and mortality across the globe. Further, generation of free radicals in brain is significantly enhanced during reperfusion and produces detrimental effects. However, various mechanisms of neuronal injury in stroke have been suggested. Thus, attempts were made with the drugs, which can intervene in these biochemical events as to prevent the cerebral injury. Few initiatives have already been taken using cell lines viz. PC12 cells, SH-SY5Y cells, human neuroblastoma, primary cultures of neuronal cells and mixed cultures of neuronal and glial cells towards developing *in vitro* models to understand the mechanisms involved in the pathophysiology of cerebral stroke. The present investigations were carried out to develop a rapid, cost effective and more sensitive mechanism based *in vitro* model of ischemic stroke as first tier of screening of neuroprotective drugs for their anti-stroke potential.

To achieve these objectives, initial experiments were carried out to optimize the various conditions for oxygen glucose deprivation (OGD) and reoxygenation using PC12 cells. Further, for validation of the *in vitro* model developed during the study, *trans* resveratrol and curcumin were used for three time schedules i.e. pre-treatment (for 24 h prior to OGD), post-treatment (for 24 h after OGD) and whole-treatment (for 24 h prior to OGD + 6 h during OGD + 24 h after OGD) at different non cytotoxic concentrations. *Trans* resveratrol and curcumin were selected as model drugs to study the validity of PC12 cells-OGD *in vitro* model, since both of them are known to have strong multiple pharmacological properties. Further, extrapolative studies were carried out using *trans* resveratrol as a test drug in rat middle cerebral artery occlusion (MCAo) model of ischemic stroke.

Experiments were carried out to identify the optimum durations of oxygen-glucose deprivation (OGD) and reoxygenation. Further, the precise role of glucose as one of the limiting factors during reoxygenation was demonstrated. The OGD of 6 h followed by a reoxygenation of 24 h with 4-6 mg/ml glucose concentrations in culture medium was found to be the optimum conditions to create the cerebral stroke like situations under *in vitro* environment using PC12 cells.

Using optimized conditions of OGD-reoxygenation and glucose concentration in culture medium during reoxygenation, studies were carried out for numbers of

endpoints involved in the process of causation and progression of cerebral stroke. Significant alterations in the oxidative stress indicator markers have been recorded, which shows the responsiveness of the system to the ischemic insult. OGD induced elevated levels of PGE_2 were also indicative that PC12 cells growing in the experimental setup have responded in the same fashion as in case of ischemic stroke. Levels of nitric oxide (NO) following OGD-reoxygenation insult were found to be increased. More than three fold increase in the accumulation of Ca^{2+} following 6 h OGD and 24 h of reoxygenation was observed.

The expression profile of genes involved in apoptosis and oxidative stress induced damages has been studied and significant responses to the ischemic insult in cultured PC12 cells have been found. Statistically highly significant decrease in the dopamine DA-D_2 receptor levels were detected in PC12 cells under the experimental conditions.

In general, a dose dependent protective potential could be observed in both pre and whole-treatment groups of *trans* resveratrol. A clear cut synergistic response was observed in whole-treatment group. This synergistic response in efficacy might be due to cumulative response of pre and post-treatment in whole-treatment group.

In case of intracellular calcium, it seems that either an additional period of pre-treatment or higher concentrations of *trans* resveratrol might increase the efficacy to restore the levels, as following the post and whole-treatment at highest concentration (25μM), the values brought to the basal level. All the treatment schedules and concentrations of *trans* resveratrol used were significantly effective in reducing PGE_2 levels in PC12 cells received 6 h of OGD and 24 h of reoxygenation. These findings confirmed that OGD-reoxygenation insult reduces the dopamine DA-D_2 bindings by 51.6% of normoxia control. Unlike rest of the endpoints studied, only pre-treatment of *trans* resveratrol was found to restore the bindings in a dose dependent manner, whereas no recovery could be seen in case of whole-treatment group.

Expression of the genes were found to drop down significantly and reached almost to the basal level following whole and pre-treatment of *trans* resveratrol. Contrary to the transcriptional (mRNA) changes, expressions at translational (protein) level were not found to be recovered significantly for any of the gene studied.

The treatment schedule of curcumin was similar as in the case of *trans* resveratrol. Except one or two endpoints, in general pre-treatment of curcumin was found to be better than other treatment schedules followed by whole-treatment group, whereas, the post-treatment group was observed minimum restorative under the experimental set up.

Though curcumin is well known for its anti inflammatory and antioxidant activities but at the same time, pro-oxidant activity of curcumin at higher doses and long exposure period has also been reported in various experimental models. In case of intracellular calcium, it is clearly seen that the lowermost concentration of curcumin used in pre-treatment was able to restore the values near to normoxia control, while higher doses in the same group can be considered as pro-oxidant.

Like *trans* resveratrol in case of curcumin also, all the treatment schedules and concentrations were found effective to control the OGD-induced alterations in PC12 cells. However, no adverse affect of higher doses could be detected as almost equal magnitude of reduction in PGE_2 levels was recorded. The findings for DA-D_2 receptor following the curcumin treatment were almost similar to the *trans* resveratrol.

Incidentally, the trend for the OGD-reoxygenation induced alteration in the expression of mRNA of studied genes known to be involved in the cascade of ischemic cerebral stroke was similar following the treatment of both curcumin and *trans* resveratrol. To the best of my knowledge, following OGD-reoxygenation insult induced changes in mRNA expression of studied genes and restoration by *trans* resveratrol and curcumin in PC12 cells have never been studied earlier. Similar to the *trans* resveratrol, recovery at protein level following curcumin treatments was non significant.

On the basis of the observations on the anti-stroke potential activity of *trans* resveratrol and curcumin, it could be concluded that PC12 cells-OGD *in vitro* model for cerebral stroke is able to mimic most of the endpoints happened during stroke and responded specifically for both the drugs tested i.e. curcumin and *trans* resveratrol. In case of *trans* resveratrol, whole-treatment group was found to be better than other treatment groups while pre-treatment of curcumin could be recorded better. In totality, curcumin has shown better protective potential than *trans* resveratrol in PC12 cells-OGD *in vitro* model under the experimental conditions. However, *trans* resveratrol

was selected for *in vivo* validation studies in rat MCAo model of cerebral stroke since studies using curcumin in same *in vivo* model have already been carried out by others.

In vivo studies were conducted using rat MCAo model of cerebral stroke to study the extrapolation of the data received through PC12 cell-OGD *in vitro* model. *Trans* resveratrol at 20 mg/kg, p.o.(per oral dose) was selected for the study. The data confirmed that the ischemic insult following 24 h and 7 days reperfusion was capable of inducing significant neurobehavioral impairment in MCAo rat model. A self recovery was also reported by 7 days of reperfusion without any drug treatment. The pre-treatment of *trans* resveratrol of seven days was found to be the best amongst the treatment groups as it significantly restored the parameters studied in time dependent manner i.e. 24 h and 7 days of reperfusion. Oxidative stress indicator markers were found to be altered following MCAo-reperfusion. A decrease in body weight following MCAo, observed in the present study, is consistent with the earlier reports of post-ischemic loss in body weight.

Interestingly, a significantly lower magnitude of antioxidant activity of *trans* resveratrol in post-treatment schedules was observed. Further, after multiple doses of post-treatment i.e. for 7 days during reperfusion period *trans* resveratrol was rather found to be causing adverse or no effects in general for the parameters studied. These findings are also in coordination with the infarction volume observed in the present investigations.

These studies would be of immense significance not only in understanding the mechanisms involved in the ischemic cerebral stroke at cellular and molecular levels, but would also be useful in adopting this PC12 cells-OGD system as a cost effective, reliable, more sensitive with better predictive values for screening of anti-stroke potential of drug candidate molecules in very rapid way. Further, this system would also be useful to extrapolate the data with rat MCAo model of ischemic stroke and to the clinical situations.

ACKNOWLEDGEMENTS

I have worked with great number of people and their contribution in assorted ways to the research and penning down thesis deserved special mention. It is a pleasure to convey my gratitude to all of them in my humble acknowledgment.

First of all I would like to record my sincere gratitude to my revered supervisor Dr. G. S. Randhawa, ,Professor, Department of Biotechnology, I.I.T. Roorkee, Roorkee, for his efficient supervision, constructive criticism, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences and exposure through out the work. His truly scientific foresightedness has made him constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student, a researcher and which a scientist look for.

My vocabulary is not wide enough to express my high sense of gratitude to Dr. A. B. Pant, Scientist, In vitro Toxicology lab, I.I.T.R., Lucknow, who hosted me in his lab for a part of my research work and subsequent helpful discussions, which made him a backbone of this research and so this thesis. His involvement with his originality has triggered and nourished my intellectual maturity that I will benefit from for a long time to come. Above all and the most needed he provided me unflinching encouragement and support in various ways. I am indebted to him more than he knows.

I am grateful to the members of my advisory committee, Dr. R. Prasad, Associate Professor, Department of Biotechnology and Dr. S. K. Sondhi, Professor, Department of Chemistry for constant help and kind cooperation throughout my thesis work.

I am heartly thankful to Prof. R. Barthwal, Prof. R. P. Singh, Dr. H. S. Dhaliwal, Dr. R. Prasad, Dr. V. Pruthi, Dr. A. K. Sharma, Dr. P. Roy, Dr. S. Ghosh, Dr. B. Chaudhary, Dr. P. Kumar, Dr. S. Tomar, Dr. R. Pathania and Dr. N. Navani, faculty members of the department for their valuable suggestions and cooperation.

I attribute my sincere thanks to Dr., Sanjay Yadav, Dr. Govind Singh and Dr. Maqsood from I.I.T.R. Lucknow, for their tremendous support.

My sincere thanks to Vivek Gupta and Durga Prasad Panigrahi for being my role model to work hard. I would like to thank Vivek for being first one who taught me work with cell lines. Words are insufficient to praise their affectionate behavior and total support through the course of this investigation. I wish them all happiness and success in their life.

Imran, Mahendra, Ritesh, Vinay, Abhishek, Smita and Anshi play instrumental in accomplishment of my mission and make my stay at Lucknow memorable. I forward my thanks to other members of my group at I.I.T.R. Lucknow for their simulative discussions on various topics during my stay.

I would like to remember and thanks Shailu, Naincy, Nidhi, Vijay and Neelam for their cooperation and like to acknowledge Praneeta, Nagesh, Swati, Shilpi, Manisha and Umesh my juniors for the help provided by them. I also want to express my gratitude to all my friends at I.I.T. Roorkee and research scholars of Biotech Department.

To make my stay pleasant in the department and support provided in completion of this work, non-teaching staffs of the department are duly acknowledged.

I would like to specially thank Payal, Meena, Sheetu, Sowmya, Preeti ,Avlokita and Shilpa to name a few, for the wonderful company they gave me, thus making my stay in Sarojini Bhawan a memorable one.

I was extraordinarily fortunate in having Pradeep Kumar Singh as my teacher and friend. I could never have embarked and started all of this without his prior teachings and guidance which has opened wide prospects and unknown areas.

Financial support from Council of Scientific and Industrial Research (CSIR), New Delhi, is greatly acknowledged.

I felt great pleasure and completeness because of my friend Maj. Tabrez who always ignited, encouraged and supported me during hard times.

Though, I have indebted to many people for their help and advice but the chief contributors undoubtedly are my parents. The entire work bears the stamps of thinking, forbearance, understanding, affection and sacrifice of my beloved mother and father. This is only their blessings, untiring efforts and sacrifices that today their dreams come true.

I am devoid of proper words to express my abounding feeling for my brother, Dr. Ashutosh Aggarwal for his constant support and selfless love, which gave me the confidence and the impetus to work hard. I would like to thank my wonderful sisters Neha and Shikha for their immutable encouragement, unstinting support and blessings.

Finally, I would like to thank everybody who was important to the successful realization of thesis, as well as expressing my apology that I could not mention them personally .

And above all, my achievements including this endeavor is due to the HELPING FORCE of NATURE and for THY I have no words to express my gratitude.

/01/2010 *(MEGHA AGRAWAL)*

CONTENTS

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF ABBREVIATIONS

AB/AM	Antibiotic/Antimycotic
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMV	Avian Mosaic Virus
bp	Base pairs
BCIP	5'-bromo-4-cloro-3'-indolyl phosphate
BSA	Bovine serum albumin
°C	Degree Centigrade
CCCP	protonophore m-chlorophenylhydrazone
COX	Cyclooxygenase
DAB	Diaminobenzidine
DCFH-DA	2',7'-dichlorodihydrofluorescin diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DTNB	5,5'-dithionitrobenzoic acid
EGTA	Ethylene glycol, O,O'-bis (2-aminoethyl) N,N,N',N'-tetra acetic Acid
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fig.	Figure
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAP	Growth associated protein
GFP	Green fluorescent protein
GLUT1	Glucose transporter 1
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
GSTs	glutathione S-transferases
h	Hour
Hsp	Heat shock proteins

HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HIF	Hypoxia inducible factor
HNE	4-hydroxy trans-2-nonenal
HRP	Horse radish peroxidase
HPRT	Hypoxanthine-phosphoribosyltransferase
IL	Interleukin
ICE	Interleukin 1-beta converting enzyme
iNOS	Inducible nitric oxide synthase
i.e.	That is
IERG	Immediate early response genes
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LPO	Lipid peroxidation
MCAo	Middle cerebral artery occlusion
MDA	Malondialdehyde
MPT	Mitochondrial permeability transition
MMP	Matrixmetalloprotease
MOPS	3-morpholinopropanesulphonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
mg/ml	Milligram per millilitre
mg/kg	Milligram per kilogram
min	Minute
ml	Millilitre
µg	Micrograms
µM	Micromolar
µl	Microlitre
NADH	Nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium
nNOS	Neuronal nitric oxide synthase
NMDA	N-methyl-D-aspartate
nm	Nanomolar
NRG-1	Neuregulin-1
NRU	Neutral red uptake

OGD	Oxygen-glucose deprivation
%	Percentage
PARP	Poly (ADP ribose) polymerase
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline containing Tween-20
PCR	Polymerase chain reaction
PC12	Pheochromocytoma cell line
PLL	Poly-L-lysine
PGE_2	Prostaglandin E_2
PMSF	Phenylmethylsulfonyl fluoride
PTGER	Prostaglandin E receptor
STAT	Signal transducer and activator of transcription
Quin-2AM	Quinoline tetracarboxylic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
rpm	Rotations per minute
TAE	Tris acetate EDTA
TBS	Tris buffer saline
TBE	Tris borate EDTA
TCA	Trichloro acetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TES	Tris EDTA saline
TRIS	Tris[hydroxymethyl]aminomethane
TRPM	Transient receptor potential cation channel, subfamily M
TTC	2,3,5-Triphenyltetrazolium chloride
WHO	World Health Organization
viz.	For example

Introduction

1. INTRODUCTION

Cerebral stroke is a neurological impairment of sudden onset lasting for more than 24 h or leading to death because of interrupted blood supply to the brain, thus vascular in origin. It is recognized as second largest cause of mortality with approximately 9.5% of total deaths and single largest cause of long-lasting disability across the globe and is a major socioeconomic stigma in the developing countries including India. The survivors of stroke manifest abrupt development of a range of neurological deficits and at times lead a crippled life.

The interrupted blood supply to the brain results in energy failure and secondary biochemical disturbances, which disturb the cellular homeostasis and subsequently lead to death of brain cells and functional impairment of some body functions. Strokes are broadly classified into two categories viz., ischemic and hemorrhagic strokes. Of the total, approximately 80% account for the ischemic stroke out of which 20% are due to small vessel thrombosis, 30% due to large vessel thrombosis and 30% due to emboli. A thrombotic stroke occurs when a blood clot forms within an artery that supplies blood to the brain. An embolic stroke occurs when a plaque fragment or blood clot travels to the brain from the heart or another artery supplying the brain.

A sudden decrease in blood flow in a particular area of the brain causes deprivation of oxygen and other nutrients. In the core part of ischemic region, non reversible necrotic cell death occurs within few minutes, while in the surrounding area called penumbra, cells become physiologically impaired but changes are reversible as cells of this region get oxygen and nutrients by partial perfusion from collateral vessels. Restoration of cerebral blood flow, even to sub-optimal level, provides an opportunity to the cells in penumbra region to recover and regain functionality.

The severity of neuronal damage and brain regions involved in the stroke is largely dependent upon many factors including duration of interrupted blood supply, artery involved, high blood pressure, heart disease, smoking, transient ischemic attacks, high red blood cell count, aging, gender, race, diabetes mellitus, prior stroke and family history of strokes. Predominantly, about 80% of stroke cases are reported due to occlusion in middle cerebral artery, responsible for blood supply to lateral part of cerebral hemispheres (regions involved in motor, language and visuo-spatial

function). The associated features of stroke assist in the localization of the lesion and also provide guidance to assess the magnitude of severity. The severity of stroke is also associated with genetic predisposition, life style and occupational & environmental exposures to the chemicals.

Most of the evidences gathered so far towards the understanding of pathophysiology of cerebral stroke are substantially based on the experimental studies. Brain injury after cerebral ischemia develops from a complex signaling cascade that leads to the early excitotoxicity induced necrotic cell death in the core region of infarction. In the surrounding penumbra region, mild excitotoxic and inflammatory mechanisms lead to delayed cell death that shows biochemical characteristics of apoptosis and can be reversed using therapeutic interventions. Among the pathophysiological changes postulated to occur as a response to ischemic insult and known to contribute in the brain injury are free radical production, excitotoxicity, disruption of sodium and calcium influx, enzymatic changes, stimulation of the inflammatory process, endothelin release, activation of platelets and leukocytes, delayed coagulation and endothelial dysfunction. Although, the above events have been reported following ischemic insult, they may be in any sequence and may behave independent to each other. Since, the cascade of events following ischemic insult and reperfusion are independent of each other and operate through various pathways, they may work synergistically to impair the body functions and lead to death of the brain cells. Thus, drug(s) that can intervene at any step in the pathway may be useful to work in a rationalized manner with case specific conditions.

In order to achieve more specific therapeutic interventions, extensive efforts have been made to study the anti-stroke potential of neuroprotectives, synthetic and natural drugs and dietary substances known to have antioxidant and anti-inflammatory activities. Massive generation of free radicals during reperfusion is also known as one among the major contributors leading to neuronal injury in ischemic cerebral stroke. Antioxidants like resveratrol (an active constituent of red wine and commonly found in high amount in grape skin), melatonin, adenosine, trolox and alpha-tocopherol have been found to be neuroprotective against neuronal injury induced by middle cerebral artery occlusion in rats. Melatonin (an antioxidant) and meloxicam (a COX-2 inhibitor) combination showed enhanced protection in middle cerebral artery occlusion model of ischemic stroke in rats. Also, herbal drugs (*Withania somnifera*, *Acorus*

calamus, curcumin) having antioxidant property were found to be neuroprotective in middle cerebral artery occlusion model of stroke in rats. Alpha lipoic acid, a universal antioxidant showed protection against reperfusion injury following cerebral ischemia and reduced the mortality rate in experimental animals.

Clinical and epidemiological studies carried out in India have shown no adverse metabolic effect of short-term anti-platelet therapy in patients of ischemic stroke and reported raised serum HDL that may contribute to cerebral protective effects. In randomized controlled double blind clinical trials, nimodipine was found to be beneficial in the recovery from acute cerebral ischemia. It has also been suggested that early onset of seizures after stroke is common and neither did it affect the outcome of prophylaxis nor did it recur even when not treated with anti-epileptics. Late onset of seizures were found less common and associated with more recurrences.

Dedicated and sustained efforts are being made by scientists and clinicians to understand the basic mechanisms involved in tissue injury following the ischemia and to develop possible prophylactic and therapeutic interventions to restore the physiological impairment and neuronal injury. Unfortunately, uniformity in the effectiveness of most of the drugs could not be recorded in different tier of screening. Despite proving effectiveness in animal models of ischemic stroke, most of the drugs have largely failed to fulfill their promise in clinical trials and currently there is only recombinant tissue plasminogen activator (rtPA), a thrombolytic agent, that the United States Food and Drug Administration (US FDA) has approved for the management of acute stroke, even though its use in ischemic stroke is controversial. Thus, the therapeutic interventions are largely based on the symptoms and empirical treatment is given by using synthetic as well as herbal antioxidants, anti-inflammatory and neuroprotectives. There is a need to improve our understanding on the pathophysiology involved in the stroke at cellular and molecular levels in precise manner using more sensitive and better experimental models and to have more reliable, rapid and cost effective screening system(s) with better predictive value(s) to screen large number of known neuroprotective agents for their anti-stroke potential.

Due to number of scientific, social and political reasons and above all, with technological advancement in last couple of decades, *in vitro* models became popular to understand the target specific mechanistic studies. The *in vitro* studies appear to be useful tool for the understanding of mechanism of cerebral stroke and its

pharmacotherapy since they are devoid of any interference by the indigenous factors. Initiatives have already been taken using cells viz., PC12 cells, SH-SY5Y cells, SK-N-AS cells, human neuroblastoma cells, C-6 glioma cells, primary cultures of neuronal cells and mixed cultures of neuronal and glial cells towards development of *in vitro* models to understand the mechanisms involved in the pathophysiology of cerebral stroke. Most of the *in vitro* studies are broadly focused to analyze the factors involved and events happening during the reoxygenation period, since prompt reperfusion of ischemic brain tissue is critical for restoring normal function. Thus, in the present investigation, attempts were made to study whether cultured PC12 cells could be a model of choice to understand the mechanisms involved in ischemic cerebral stroke and whether the data would be extrapolative to rat MCAo model of cerebral stroke. The PC12 cells-OGD *in vitro* model, developed during the study, was used to evaluate its responsiveness for endpoints involved in the pathophysiology of ischemic stroke following the exposure of known neuroprotectants viz., *trans* resveratrol and curcumin. These cells were employed in the study since they are most frequently used neuronal cells derived from sympathetic nerve and have been found successful over the years to study a range of neuronal functions and prominent expression of variety of neurotransmitter receptors in them. Curcumin and *Trans* resveratrol were selected as model drugs to study the validity of PC12 cells-OGD *in vitro* model since both of them are known to have multiple pharmacological properties including antioxidant, anti-inflammatory and anti-apoptotic activities and are well accepted neuroprotective drugs in Indian system of medicine.

These studies would be of immense significance in not only understanding the mechanisms involved in the ischemic cerebral stroke at cellular and molecular levels, but would also be useful in adopting the PC12 cells-OGD system as a cost effective, reliable and more sensitive model with better predictive values for screening of anti-stroke potential of drugs. Further, this system would also be useful to extrapolate the data with rat MCAo model of ischemic stroke to the clinical situations.

Taking the above into account, the aims and objectives of the present study were:

- Development and validation of *in vitro* model of cerebral stroke using neuronal cells under ischemic (oxygen and glucose deprivation) conditions.

- Evaluation of anti-stroke potential of *trans* resveratrol and curcumin.
- Extrapolation of *in vitro* data with *in vivo* middle cerebral artery occlusion model of stroke in rat.

Review of Literature

2. REVIEW OF LITERATURE

Disease prevalence

Cerebral stroke is recognized as second largest cause of mortality with approximately 9.5% of total deaths and single largest cause of long-lasting disability across the globe and poses major socio-economic stigma in the developing countries like India (WHO, 2006).

Higher stroke mortality in United States has long been evidenced among blacks and residents of southeastern states. A greater proportion of blacks live in the southeastern states that make up the so-called stroke belt than elsewhere in the country; however, variations in socioeconomic characteristics and risk factors have also been associated with disparities in stroke, region and race (Centers for Disease Control and Prevention, 2005). In United States more than seven lac new cases of strokes occur annually. Stroke is also the leading cause of disability with an estimated 4 million stroke survivors living with stroke related deficits in US alone. More than 70% of stroke survivors remain vocationally impaired, more than 30 % require help for activities of daily living, and more than 20 % walk only with assistance (Tuhrim, 2002). The overall prevalence of stroke is 2.77% (men 3.72% and women 1.77%) in Asian Indians living in the United States (Baweja *et al.*, 2004; Pandian *et al.*, 2007).

Although, reporting of cerebral stroke in India is very low due to number of reasons, attempts are being made to enumerate the exact number of victims, so that the magnitude of the disease could be assessed. Preliminary epidemiological data has revealed that the prevalence of stroke is about 0.2% in Indian population over 20 years of age with a mortality rate of 1.2% (Anand *et al.*, 2001, Dalal, 2002; Pandian *et al.*, 2007). Surprisingly, in India the incidences of stroke are higher in young than in the West, but the etiology behind this changed ratio is not well defined (Jayakumar *et al.*, 1991; Anand *et al.*, 2001; Gulati and Kalra, 2003; Pandian *et al.*, 2007).

Risk factors involved

As per the recommendations of WHO, stroke is a "neurological impairment of vascular origin having sudden onset and lasting for more than 24 h or leading to death because of interrupted blood supply to the brain" (WHO, 2006).

Ischemic stroke occur when an artery supplying blood to the brain becomes blocked, suddenly decreasing or stopping blood flow and ultimately causing brain cells

to die and some body functions to become impaired. Strokes are broadly classified into two categories viz., ischemic and hemorrhagic strokes. Of the total, approximately 80% accounts for ischemic stroke, out of which 20% are due to small vessel thrombosis, 30 % are due to large vessel thrombosis and 30% due to emboli (Pandian *et al.,* 2007). A thrombotic stroke occurs when a blood clot forms within an artery that supplies blood to the brain. An embolic stroke occurs when a plaque fragment or blood clot travels to the brain from the heart or another artery supplying the brain (Elkind, 2003). Hemorrhagic stroke (20%) occurs when a blood vessel in the brain ruptures. Long-term hypertension can weaken the walls of blood vessels in the brain and eventually cause a hemorrhagic stroke (cerebral hemorrhage). Hemorrhagic strokes are divided into two types, intra cerebral hemorrhage (15%) and subarachnoid hemorrhage (5%). An intra cerebral hemorrhage is characterized by bleeding into the brain itself. High blood pressure may cause small blood vessels to bulge and eventually burst spilling blood into the brain. The bleeding damages brain cells mainly due to pressure effect, so that the damaged area cannot function properly. Subarachnoid hemorrhage is characterized by bleeding into an area below the arachnoid membrane (Gorelick, 1986; Mergenthaler *et al.,* 2004; WHO, 2006; Misra *et al.,* 2009).

Risk factors for cerebral stroke include both genetic predisposition and environmental factors, which can be a consequence of natural processes or results from a person's lifestyle. Risk factors that can be influenced include hypertension, heart disease, smoking, diabetes mellitus, transient ischemic attacks and high red blood cell count. Risk factors that cannot be modified include aging, gender, race, prior stroke and a family history of strokes (Allen and Bayraktutan, 2008). Other risk factors for stroke that can be modified include high blood cholesterol and lipids, reduced physical activity and obesity (Tan *et al.,* 2004; Khan *et al.,* 2005; Allen and Bayraktutan, 2008). Demographic changes that may also be associated with risk factors of stroke as in the younger generation in Asian region includes hypertension, hypercholesterolemia, hypertriglyceridemia, smoking and diabetes (Liu *et al.,* 2002) whereas in Western population, oral contraceptives, alcohol and illicit drug use are more frequent risk factors (Tegos *et al.,* 2000; Leys *et al.,* 2002). Cardio-embolic stroke and atherosclerotic occlusive disease have been considered as the most common cause of ischemic stroke (Mehndiratta *et al.,* 2004). Studies have also shown that moderate

consumption of alcohol is protective against stroke whereas heavy alcohol consumption increases the risk of stroke (Emberson and Bennett, 2006).

Stroke and brain regions involved

The sites of the brain involved in stroke depend upon the severity, duration stoppage of blood supply to the brain and the artery involved during the stroke. Eighty percent of strokes occur in the territory of middle cerebral arteries (MCAo) that supply the lateral part of the cerebral hemispheres, including the brain regions responsible for motor, language and visuospatial function (Keith *et al.,* 1997). A large number of clinical syndromes have been described, but in approximately 70% of strokes there is limb weakness. Limb weakness may result from stroke affecting any part of the motor pathway from the cerebral cortex (prefrontal gyrus) to the internal capsule, to the pyramidal (corticospinal) tracts as they traverse the brainstem (Roh and Lee, 1996; Krakauer, 2005).

Associated features of stroke assist in the localization of the lesion and may also provide a guide to the severity of stroke, e.g., isolated hemiparesis usually arises from sub cortical infarction affecting the internal capsule because of small vessel occlusive disease (lacunar stroke), but the combination of hemianopia, hemisensory loss, and dysphasia with hemiparesis signifies a proximal MCA occlusion with extensive infarction of the dominant cerebral hemisphere (Gupta *et al.,* 2004a; Berger *et al.,* 2008).

Stroke etiology

Stroke occurs when blood flow to the brain is interrupted by either a blocked or burst artery, resulting in a sudden decrease in the blood flow to an area of the brain, depriving brain cells of oxygen and other nutrients. Ischemia develops within minutes, forming two zones around the site of thrombosis or embolism (Kobayashi and Mori, 1998). Brain cells at the center of ischemic region, where circulation is completely arrested, suffer irreversible damage in several minutes. However, in cells surrounding the center (penumbra), ischemia is partial due to the presence of perfusion from collateral vessels (Astrup *et al.,* 1981). In this region blood flow is reduced to levels below the threshold for electrical failure and above the threshold for energy failure (Mergenthaler *et al.,* 2004). Restoration of cerebral blood flow, even to a sub-optimal level, provides an opportunity for those brain cells to recover and regain functionality (Hossmann, 1994; Patro *et al.*, 1999).

Pathophysiology involved in stroke

The current pathophysiological understanding of stroke is substantially based on experimental studies. Brain injury after cerebral ischemia develops from a complex signaling cascade that evolves in a partially unraveled spatiotemporal pattern. Early excitotoxicity can lead to acute necrotic cell death, which forms the core of the infarcted area. The penumbra that surrounds the infarct core suffers milder insults due to mild excitotoxic and inflammatory mechanisms leading to delayed cell death having biochemical resemblance to apoptosis (Mergenthaler *et al.,* 2004). Among the pathophysiological changes that are postulated to occur as a response to stroke are free radical production, excitotoxicity, disruption of sodium and calcium influx, enzymatic changes, stimulation of the inflammatory process, endothelin release, activation of platelets and leukocytes, delayed coagulation and endothelial dysfunction. All of these pathophysiological reactions may contribute to the brain injury following the onset of stroke (Scott and Gray, 2000; Cheung, 2003; Jordan *et al.,* 2007; Moustafa and Baron, 2008).

Energy failure: Brain tissue has a relatively high consumption of oxygen and glucose and depends almost exclusively on oxidative phosphorylation for energy production. Focal impairment of cerebral blood flow restricts the delivery of substrates, particularly oxygen and glucose, and impairs the energetics required to maintain ionic gradients (Martin *et al.,* 1994; Kuroiwa *et al.,* 2000; Jordan *et al.,* 2007). Within 2 min of stroke onset, neurons and glia in brain regions most deprived of blood (the ischemic core) undergo a sudden and profound loss of membrane potential caused by failure of the Na^+/K^+ ATPase pump. This anoxic depolarization represents a collapse in membrane ion selectivity that causes acute neuronal injury because neurons cannot survive the energy demands of repolarization while deprived of oxygen and glucose (Anderson *et al.,* 2005). At the same time, the energy dependant processes, such as presynaptic reuptake of EAA, are impeded, which further increase the accumulation of glutamate in the extracellular space (Grewer *et al.,* 2008).

Excitotoxicity: Excitotoxicity is based on the release of excitatory amino acids mainly glutamate which plays an important role. It has long been accepted that excitotoxicity due to high concentrations of glutamate can damage the neurons (Hoyte *et al.,* 2004; Grewer *et al.,* 2008). The sequence of event starts with a decrease in cerebral blood flow that leads to energy failure causing diminished Na^+/K^+ exchange. As the cell is

not able to maintain its membrane potential, it depolarizes triggering calcium influx through voltage sensitive calcium channels. This further depolarizes the cell membrane and stimulates the release of massive amounts of the excitatory neurotransmitter glutamate into the extracellular space (Grewer *et al.,* 2008).

Neurons are normally exposed to only brief pulses of glutamate because most of the glutamate is actively re-uptaken by the presynaptic terminals and glial cells. During ischemia, the energy-dependant mechanisms responsible for glutamate reuptake are impaired; hence extracellular glutamate levels are enhanced. This elevation of glutamate level causes prolonged and excessive activation of membrane glutamate receptors (Dirnagl *et al.,* 1999; Nishizawa, 2001). Glutamate receptors can be divided into two types i.e., ionotropic and metabotropic. The ionotropic receptors are ligand-gated ion channels while the metabotropic receptors are linked via G-proteins to the cAMP and IP_3 second messenger systems (Grewer *et al.,* 2008). The ionotropic receptors are subdivided into three receptor classes that are named according to their selective agonists: AMPA receptors, kainate receptors and NMDA receptors (Small *et al.,* 1999). AMPA and kainate receptors are activated first and trigger rapid excitatory neurotransmission by promoting entry of Na^+ into neurons leading to depolarisation of the post-synaptic terminals. Persistent depolarization leads NMDA receptors to relinquish their magnesium block thus causing excessive entry of calcium through the NMDA receptors. Increase in intracellular calcium triggers calcium dependant processes which culminate in secondary injury and eventually cell death (Small and Buchan, 1996; Mehta *et al.,* 2007).

Calcium overload: Calcium ions play important role in the organization of cell function as intracellular messengers and regulators of neurotransmitter release, electrical activity, cytoskeleton function, cell metabolism and gene expression (Triggle, 1994). In brain ischemia, gating of postsynaptic glutamate receptors and other membrane channels triggers intracellular Ca^{2+} overload and cell death (Bano *et al.,* 2005). In excitotoxic settings, the initial Ca^{2+} influx through glutamate receptors is followed by a second uncontrolled Ca^{2+} increase that leads to neuronal death (Mehta *et al.,* 2007; Grewer *et al.,* 2008). Cytosolic Ca^{2+} rises as a result of net entry of Ca^{2+} across the plasmalemma or transiently due to liberation of Ca^{2+} from intracellular stores. In ischemia, early increase in total cellular Ca^{2+} is also reported via NMDA receptors. The early influx of Ca^{2+} via NMDA receptors, probably due to the profound

ATP decrease and resulting receptor dephosphorylation, is thereafter replaced by influx through other pathways or release from intracellular stores (Lobner and Lipton, 1993). The steady-state increase in cytosolic Ca^{2+} during ischemia is largely due to Na^{+} entry and resultant activation of $2Na^{+}/Ca^{2+}$ transporter-mediated efflux from mitochondria (Mattson, 2007).

There is also an eclectic body of evidence that cytosolic Ca^{2+} levels are also elevated in the post ischemic period, which may be responsible for the calcium-dependent NOS activation, calpain activation, generation of free radicals via phospholipid metabolism and mitochondrial damage (Dugan *et al.,* 1995). Mitochondrial calcium accumulation and oxidative stress can trigger the assembly (opening) of a high-conductance pore in the inner mitochondrial membrane. The mitochondrial permeability transition (MPT) pore leads to a collapse of the electrochemical potential for H^{+}, thereby arresting ATP production and triggering production of reactive oxygen species. Calcium may also be involved in up regulation of damaging genes during and after ischemia (Sims and Anderson, 2002; Mattson, 2007).

Free radical generation and oxidative stress: Free radicals (O_2^{-}, NO_2^{-}, H_2O_2 and OH^{-}) are highly reactive molecules generated predominantly during cellular respiration and normal metabolism. An imbalance between cellular production of free radicals and the cellular defense mechanisms is referred to as oxidative stress. After brain damage induced by ischemic stroke or reperfusion, production of reactive oxygen species is known to increase drastically, leading to tissue damage via several cellular and molecular pathways (Mehta *et al.,* 2007). The increase in production of free radicals occurs through several different cellular pathways including calcium, activation of phospholipases, nitric oxide synthase, xanthine oxidase and the Fenton and Haber-Weiss reactions by inflammatory cells, leading to tissue damage (Doyle *et al.,* 2008). The severity of damage is dependent on the extent of weakening of cellular antioxidant defense mechanisms after ischemic stroke (Schaller, 2005).

Oxidative damage does not occur in isolation but participates in the complex interplay between excitotoxicity, apoptosis and inflammation in ischemia and reperfusion (Barone and Feuerstein, 1999; Doyle *et al.,* 2008). Free radical generation also occurs during the time of reperfusion. Reoxygenation during reperfusion causes an increase in cellular oxygen to levels that cannot be utilized by the mitochondria

under normal physiological conditions. As a result there is perturbation of the antioxidant defense mechanisms resulting in overproduction of oxygen radicals, inactivation of detoxification systems, consumption of antioxidants and failure to replenish antioxidants in the ischemic brain tissue (Kuroda and Siesjo, 1997; Doyle *et al.,* 2008). Under compromised state of defense mechanisms at cellular level, it induces the production of free radicals, leads to oxidation of lipids, proteins, and nucleic acids and finally alters the cellular function in a critical way (Lewen *et al.,* 2000). It is also reported that the pharmacological up-regulation of free radical scavenging enzymes can reduce infarct volumes (He *et al.,* 1993; Doyle *et al.,* 2008).

Inflammatory responses: Ischemic stroke also triggers an inflammatory reaction that progresses for days after its onset. Such an inflammatory reaction also contributes to the late stages of ischemic injury and results in worsening of neurological outcome (Doyle *et al.,* 2008). The mechanism(s) by which inflammation contributes to cerebral damage is not well understood. Alterations in the microcirculation after ischemia/reperfusion include disruption of the blood-brain barrier, edema and swelling of perivascular astrocyte foot processes, decrease in arteriolar endothelium-dependent relaxation & reduced inward-rectifying potassium channel function, altered expression of proteases & matrix metalloproteinases, increased inflammatory mediators and inflammation (Takahashi and Macdonald, 2004; Mehta *et al.,* 2007; Doyle *et al.,* 2008).

Leukocyte adhesion, predominantly to the endothelium of the venules, produces microvascular plugging, slows the rate of blood flow, and may aggravate tissue damage (Ritter *et al.,* 2000). Also, the activated astrocytes, microglia, leukocytes and endothelial cells begin to produce cytokines that are important intracellular proteins and act as signals to induce the inflammatory response, further increasing the cerebral ischemia. In experimental animals, brain ischemia induces expression of inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β (Barone and Feuerstein, 1999). In human subjects, increase in systemic production of cytokines following ischemic injury has also been demonstrated (Kostulas *et al.,* 1999; Pelidou *et al.,* 1999; Planas *et al.,* 2006). These cytokines are known to attract leukocytes and up-regulate the expression of adhesion molecules such as intracellular adhesion molecule-1, selectins and integrins on endothelial cells and leukocytes (Mehta *et al.,* 2007; Wang *et al.,* 2007; Mousa, 2008).

Studies have provided evidences that expression of inflammation related enzymes - inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) - are critical mechanisms by which inflammatory cells influence the progression of cerebral ischemic damage. In the brain, iNOS is induced during post ischemic inflammation after transient or permanent middle cerebral artery occlusion in rodents. Inducible NOS mRNA is up-regulated and peaks at 12-48 h after ischemia (Iadecola *et al.,* 1995; Ross and Iadecola, 1996; Basu *et al.,* 2005). The increase in iNOS is associated with increase in iNOS enzymatic activity and nitric oxide production as revealed by accumulation of nitric oxide (Hirabayashi *et al.,* 2000). Inducible NOS is induced in neutrophils infiltrating the injured brain and in cerebral blood vessels in the ischemic territory. The presence of iNOS in human brain after ischemic stroke has also been reported using immunohistochemical localization (Forster *et al.,* 1999; Murphy and Gibson, 2007). The data with iNOS inhibitors in null mice have suggested that the nitric oxide produced by iNOS is an important factor in the delayed progression of ischemic damage and can be targeted (Murphy and Gibson, 2007; Doyle *et al.,* 2008).

Nitric oxide is a ubiquitous free radical that is involved both in normal cellular function and in pathological processes. Nitric oxide is generated from L-arginine by NOS, a family of enzymes that are present constitutively in endothelial cells and neurons (endothelial and neuronal NO synthases). A third isoform iNOS is not present in most cells but its expression is induced during development and in pathological states, most characteristically those associated with inflammation. Inducible NOS generates toxic levels of nitric oxide and is thought to contribute to the cytotoxicity induced by inflammation (Forster *et al.,* 1999; Murphy and Gibson, 2007). The mechanisms by which nitric oxide and its derived species exert their cytotoxic effect are diverse and includes oxidative stress mediated DNA damage and inhibition of enzymes involved in the synthesis of ATP (Iadecola, 1997).

Cyclooxygenase (COX) and stroke: COX is a rate-limiting enzyme in the synthesis of prostaglandins and thromboxanes. Two isoform have been described: COX-1 and COX-2. COX-1 is constitutively expressed in many cells where it produces prostanoids that are involved in normal cellular function (Vane and Bakhle, 1998). COX-2 is normally expressed at low levels in some cells such as neurons (Tomimoto *et al.,* 2002). However in many cells COX-2 is up regulated in response to mitogens, inflammatory mediators and hormones (Xiao *et al.,* 2005). In models of inflammation,

COX-2 is up-regulated and contributes to tissue damage through a production of reactive oxygen species (O'Banion, 1999; Tomimoto *et al.,* 2002). Superoxide produced by COX-2 activity may react with the nitric oxide to form powerful oxidant NO_2. There is evidence that COX-2 participates in the cascade of events involving cerebral ischemia; COX-2 mRNA and protein expression are up-regulated 12-24 h after cerebral ischemia in rodents (Nakayama *et al.,* 1998). COX-2 expression in rodents is observed in neurons at the periphery of the infarct, in vascular cells, and possibly in microglia (Nogawa *et al.,* 1997). COX-2 immunoreactivity has also been reported in ischemic neurons at the border of ischemic territory and in neutrophils and vascular cells (Nakayama *et al.,* 1998). The up-regulation in COX-2 immunoreactivity is confined to the area of damage (Hara *et al.,* 1998; Iadecola *et al.,* 1999).

Administration of NS-398, a relatively selective COX-2 inhibitor 6 h after ischemia reduced the infarct size by 20-30% in a model of focal ischemia in rats (Nagayama *et al.,* 1999). Involvement of COX-2 in the late stages of ischemic injury has been demonstrated using delayed administration of COX-2 inhibitor NS-398, which reduced the infarct size significantly (Hara *et al.,* 1998). Post stroke onset administration of aspirin, a known COX inhibitor and antiplatelet aggregator, has been suggested a good neuroprotective (Berger *et al.,* 2004).

Impairment of blood brain barrier: The integrity of the blood brain barrier (BBB) depends on the interaction of the cellular matrix, which is composed of endothelial cells and astrocytes. Because of cerebral ischemia, this cellular matrix and the intercellular signal exchange is damaged. Proteases, especially matrix metalloproteases (MMPs) play an important role at this. MMP2 and MMP9 are induced 1-3 h after cerebral ischemia. The expression of MMP correlates with the damage to the BBB, the risk of a hemorrhagic transformation and the extent of neuronal damage (Offen *et al.,* 2004; Kurzepa *et al.,* 2005). Destruction of basal lamina by MMPs permits immigration of leukocytes and leads to a vasogen edema (Sandoval and Witt, 2008). Inhibition of MMPs not only reduces the damage to the BBB but also reduces infarct size (Rosenberg *et al.,* 1996; Asahi *et al.,* 2001; Sandoval and Witt, 2008).

Programmed cell death: The process of apoptosis refers to programmed cell death and involves activation of specific intracellular pathways that have evolved to produce the controlled destruction of the cell (Nicotera and Lipton, 1999; Doyle *et al.,* 2008). It results from DNA cleavage and by other autotypic processes causing nuclear

shrinkage, chromatin clumping, and ultimately cell death (Portera-Cailliau *et al.,* 1997).

Following cerebral ischemia, brain cells can die either of necrosis or apoptosis (Graham and Chen, 2001; Freeman and Schellinger, 2006; Doyle *et al.,* 2008; Nakka *et al.,* 2008). It is now established that apoptotic/necrotic cell death in the brain following cerebral ischemia is dependent on the nature of the stimulus and the type of cells involved. In the nervous system, genes have been identified which either (i) promote apoptosis: Bax, c-fos, c-jun, and ICE-like proteases or (ii) block apoptosis: Bcl-2 and Bcl-xL. The genes that suppress or augment cell death are expressed at higher levels and are activated in both the early and late phases of ischemia (Mattson *et al.,* 2000; Nakka *et al.,* 2008). The expression of immediate early response genes (IERG), c-Fos and c-Jun together with other IERG were demonstrated in numerous *in vivo* experiments (Kinouchi *et al.,* 1994; Neumann-Haefelin *et al.,* 1994), but only few reports are available demonstrating the expression of c-Fos and c-Jun after *in vitro* ischemia in cell culture model (Prabhakar *et al.,* 1995; Ness *et al.,* 2008 Ginet *et al.,* 2009). MK 801, a noncompetitive NMDA receptor antagonist, was found to significantly reduce the expression of c-Fos and c-Jun mRNA in rat neuronal cultures, whereas no effect was observed in PC12 cells (Gerlach *et al.,* 2002).

Under ischemic conditions leading to apoptosis, the predominance of pro-apoptotic factors leads to the release of protein from the inter membrane space into the cytoplasm of which cytochrome-C has been characterized. In the cytoplasm, cytochrome-C interacts with a protein caspase-9 to activate caspase-3. This produces events including the activation of the caspase dependant DNAase leading to fragmentation of DNA and thereby cell death (Sims and Anderson, 2002; Fiskum, 2000). Zhu *et al.* (2004) have suggested the expression of 76 predominant genes in hypertensive tissues of rat model of ischemic stroke and their role in the progression of disease through separate pathophysiological mechanisms at pre-transcriptional, transcriptional and post-transcriptional levels.

Neuregulin-1 (NRG-1) is expressed throughout the immature and adult central nervous system, and it has been demonstrated to influence the migration of a variety of cell types in developing brain. Elevated levels of NRG-1 transcript are found in the adult brain after injury, leading to the suggestion that NRG-1 is involved in the physiological response to neuronal injury. Experimental rats pre-treated with NRG-1

protein had shown increased motor performance and less cerebral infarction post ischemic stroke in comparison to untreated rats (Shyu *et al.,* 2004).

Heat shock proteins (Hsps): Heat shock proteins are among the most conserved proteins known to be expressed in almost all the organs in both plants and animals including human beings. Since the expression of these proteins gets altered due to range of stimuli like ischemia, seizures, heavy metal poisoning and endotoxins, therefore, they are now also known as stress proteins.

In the nervous system, Hsps are induced in a variety of pathologic conditions including cerebral ischemia, neurodegenerative disorders, epilepsy and trauma. Expression is detected in a variety of cells within the nervous system including neurons, glia and endothelial cells. It is observed in the brain within several hours of the insult and persists for a few days (Foster and Brown, 1997; Brown, 2007). There is increasing body of evidence that a significant accumulation of Hsp72, Hsp70 and Hsp27 mRNAs occurs following cellular ischemia (Massa *et al.,* 1996; Mehta *et al.,* 2007). Advances in molecular biology has made it possible to selectively over-express Hsps by *in vivo* gene transfer to neurons with an HSV vector over expressing the inducible Hsp72 or using transgenic animal strains (Hoehn *et al.,* 2001). Under an *in vitro* model of cerebral ischemia, astrocytes cultures receiving the Hsp72 expressing vectors survived against the insults better than the control (Lee *et al.,* 2001). Since Hsp72 is induced as a result of cerebral ischemia and protects the cells, it has been postulated that modulation of Hsp72 can be a beneficial approach. However, viral gene therapy is not practical and therefore other pharmacological methods of inducing Hsp are being investigated. Inducible HSPs also prevent protein aggregation during exposure to physiochemical insults such as elevated temperature, activated oxygen and nitrogen intermediates, inflammatory mediators or infection. During ischemia, stress proteins may enhance cell survival by preventing protein aggregation or facilitating refolding of partially denatured proteins (Volobоueva *et al.,* 2008). Suitability of small peptides especially the synthetic peptides like CMX-9236 in neuroprotection has been suggested via an activation of a beneficial signal transduction pathway in both *in vitro* and *in vivo* models of cerebral ischemia (Shashoua *et al.,* 2003).

Genetic predisposition: In an epidemiological study on two different races of human being, existence of genetic predisposition to stroke development was demonstrated. However, presence of other predisposing risk factors and genetic heterogeneity of

human populations may also play an important role. In particular, structural alterations of the genes are consistently present in diseased individuals, suggesting an important role of mutation-dependent mechanisms in stroke predisposition (Ross *et al.,* 2007; Tonk and Haan, 2007). Further, the stroke-prone spontaneously hypertensive rats have provided valuable information on genetic factors involved in stroke predisposition. Among them, the genes-encoding natriuretic peptides have been identified as genes involved in stroke prone spontaneously hypertensive rats (Brosnan *et al.,* 1999). Finally, the use of intermediate disease phenotypes provides a reductionist approach that may contribute to important accumulating information on genes contributing to cerebrovascular accidents (Rubattu *et al.,* 2004; Ross *et al.,* 2007).

Current strategies for therapeutic interventions in stroke

Till date, recombinant tissue plasminogen activator (rtPA), a thrombolytic agent, is the only FDA approved drug available for stroke therapy (Fisher and Bastan, 2008). However, its use in ischemic stroke is still controversial (Liang *et al.,* 2008). The early use of aspirin has also been suggested to reduce stroke recurrence (Sandercock *et al.,* 2008) and clopidogrel monotherapy is considered as reasonable alternative for patients having sensitivity for aspirin (Kirshner, 2008) as it has efficacy and safety profiles similar to aspirin (Belvis *et al.,* 2008). Presently the treatment of stroke involves four major approaches: (i) rapid recanalization of occluded vessels so as to restore the blood flow to ischemic area, (ii) improvement of microcirculation in the ischemic penumbra by antithrombotic or antiplatelet agents, (iii) to interrupt the biochemical cascade of ischemic brain injury in order to prevent neuronal death (neuroprotection), and (iv) replacement of dead cells by differentiated stem cells. With past experience, drugs having multimodal action through various pathways are being tested for their clinical efficacy against early onset of stroke. Albumin, hematopoietic growth factors and citicoline are few promising candidate drug molecules that are currently under clinical trial (phase III) and may be adopted as a drug of choice to cure the stroke in near future (Minnerup and Schabitz, 2009).

i. Rapid recanalization of occluded vessels

Intravenous thrombolytic therapy: Even after some disappointing results in earlier days (Alexandrov *et al.,* 1997; Brockington and Lyden, 1999; Hacke *et al.,* 1999), experimental evidences finally led to the commercialization of first and only FDA approved thrombolytic agent i.e., rtPA for therapy against acute ischemic stroke

(Asahi *et al.,* 2000; Fisher and Bastan, 2008) and to reduce the hemorrhagic risk profiles. Large numbers of new generation thrombolytics are at various stages of development like monteplase, tisokinase, pamitelpase, desomoteplase, tenecteplase and alteplase (Verstraete, 1999; Verstraete, 2000; Tanne and Levine, 2008; Meretoja and Tatlisumak, 2008).

Intra-arterial thrombolytic therapy: Local intra-arterial thrombolysis performed with a microcatheter that is placed into, beyond and proximal to an arterial occlusion is now used worldwide (Lewandowski *et al.,* 1999). Attempts are being made using urokinase, tPA and pro-urokinase (Roberts *et al.,* 2002; Xavier *et al.,* 2003). In a randomized clinical trail on patients with onset of stroke less than 6 h, pro-urokinase resulted in 66% recanalization rate as compared to 18% with heparin treated group (Del Zoppo *et al.,* 1998; Kase *et al.,* 2001). Intra-arterial thrombolytic therapy may result in rapid clot lysis and higher recanalization rates as compared to the intravenous therapy (Xavier *et al.,* 2003).

ii. Improvement of microcirculation in the ischemic penumbra

Extensive studies have been conducted to study the antithrombotic activity of heparin and its analogues like nadoparin, flaxiparin and danaproid in experimental models of acute ischemic stroke as well as in patients of stroke. Unfortunately, the data obtained was not convincing to suggest them as remedial approach for the treatment of stroke (Sherman, 1998; Munoz-Torrero and Diez-Tejedor, 1999; Berge and Sandercock, 2002; Bath *et al.,* 2000; Wong *et al.,* 2007). A number of agents including abciximab, tirofiban, eptifibatide (Mandava *et al.,* 2008), fibrinogen antagonist having platelet aggregation reducing capacity were studied for possible therapeutic intervention in the stroke, but none of them have reached to the clinical level (Winkley and Adams, 2000; Mandava *et al.,* 2008).

iii. Neuroprotection

Neuroprotection remain one of the most exciting therapeutic ways to protect ischemic neurons in the brain from irreversible injury especially to core and penumbra regions. With better understanding of the cascade of events occurring during stroke, a number of therapeutic interventions have been tried. These approaches included the use of various calcium channel blockers viz., nimodipine, felodipine, isradipine, flunarizine, darodipin, SNX- 111 (Buchan *et al.,* 1994; Yenari *et al.,* 1996; Perez-pinzon *et al.,* 1997; Horn and Limburg, 2001; Horn *et al.,* 2001; Levy *et al.,* 2004;

Ginsberg, 2008); NMDA receptor antagonists viz., selfotel, dextrometorfan, dextrofan, apteganel (Albers *et al.,* 1995; Lees, 1997; Davis *et al.,* 2000; Ginsberg, 2008); AMPA receptor antagonists viz., NBQX, YM90K, LY326325, ZK200775 (Teelken and De Keyser, 2002; Kundrotiene *et al.,* 2004; Iwasaki *et al.,* 2004; Ginsberg, 2008); sodium channel blockers viz., sipatrigine, crobenetine, irampanel hydrochloride (Muir *et al.,* 1998; Muir *et al.,* 2000; Carter *et al.,* 2000; Feigin, 2002; Williams *et al.,* 2003; Callaway *et al.,* 2004; Ginsberg, 2008); potassium channel opener viz., BMS-204352 (Gribkoff *et al.,* 2001; Cheney *et al.,* 2001; Jensen, 2002; Liu *et al.,* 2003; Farkas *et al.,* 2005; Ginsberg, 2008); glycine antagonist viz., gavestinel (Lees *et al.,* 2000; Sacco *et al.,* 2001; Ginsberg, 2008); polyamine antagonist viz., elprodil (Lees, 1997; Ginsberg, 2008). Although, many of these drugs are being used in the treatment of other diseases, they could not be recommended for clinical use in stroke therapy due to their failure at various stages during experimental or clinical evaluation (Ginsberg, 2008).

Besides these, a number of other approaches have also been tried like use of anti-inflammatory drugs viz., p-38 MAPK inhibitors (SB-239063 and VX-745), cyclosporine-A, methylprednisolone, (Legos *et al.,* 2001; Barone *et al.,* 2001; Yu *et al.,* 2004; Borlongan *et al.,* 2005); NOS inhibitors viz., AR-R 17477, 1,2-trifluoromethylphenyl imidazole (TRIM) and BN 80933 (O'Neill *et al.,* 2000; Haga *et al.,* 2003; Ding-Zhou *et al.,* 2003). Although the role of these anti-inflammatory agents have yet not been firmly established in the therapy of stroke, their use since the start of inflammatory events in the causation and progression of disease is well established (Yamada *et al.,* 2008; McCombe and Read, 2008). The role of 5-HT receptor agonist repinotan (Legos *et al.,* 2002; Berends *et al.,* 2005) has also been explored as a potential therapeutic tool. However, like others, this approach is also under clinical trial and yet has not been accepted for clinical use.

Growth factors as neuroprotectants: The growth factors - basic fibroblast growth factor (bFGF), osteogenic protein-1 (OP-1), vascular endothelial growth factor (Vegf), erythropoietin (EPO), and granulocyte colony stimulating factor (G-CSF) all show potential usefulness in animal models of acute stroke and stroke recovery. Two of these factors, bFGF and EPO have reached the stage of human clinical trials for the treatment of acute stroke (Ren and Finklestein, 2005; Bani-Yaghoub *et al.,* 2008). It has also been reported that glial cell-derived neurotrophic factor (GDNF)-fibrin glue

has the capability to reduce the total infarct size and to improve motor deficits (Cheng *et al.,* 2005). Intranasal NGF was found to reduce infarction after passing through blood brain barrier and could be suggested as promising agent for the treatment of stroke (Zhao *et al.,* 2004).

Synthetic drugs: For past few decades, dedicated attempts are being made to treat stroke using various approaches and many synthetic drugs have been tested at large. Here under are listed some of the promising drugs, which have been studied in last few years for their anti-stroke potential:

Citicholine	D'orlando and Sandage, 1995; Clark *et al.,* 1997, 1999, 2001; Adibhatla *et al.,* 2002
Ebselen	Yamaguchi *et al.,* 1998; Parnham and Sies, 2000; Green and Ashwood, 2005
Edaravone	Anzai *et al.,* 2004; Shichinohe *et al.,* 2004; Otani *et al.,* 2005
EGb-761	Diamond *et al.,* 2000; Paganelli *et al.,* 2006; Saleem *et al.,* 2008
Endothelin	Andrasi *et al.,* 2002; Bajory *et al.,* 2002; Lehmberg *et al.,* 2003; Fisher and Brott, 2003; Baamonde *et al.,* 2004.
Melatonin	Sinha *et al.,* 2001; Kilic *et al.,* 2004; Kilic *et al.,* 2005
NXY-059	Kuroda *et al.,* 1999; Zhao *et al.,* 2001; Peeling *et al.,* 2001; Marshall *et al.,* 2001; Green and Ashwood, 2005
TAK-044	Ikeda *et al.,* 1994; Watanabe *et al.,* 1995; Briyal *et al.,* 2006, 2007
Tirilazad mesylate	Bath *et al.,* 2000; Johnston *et al.,* 2002; Green and Ashwood, 2005

Herbal drugs: Based on the Indian system of medicine (Ayurveda) large numbers of herbal drugs are being used clinically for thousands of years though their exact mechanism of action is not known. But, in last few years, a reverse pharmacological approach has been adopted to generate laboratory data on their mechanism of action on the basis of their known therapeutic potential for various diseases. In this connection, several plants and their products have also been experimentally and clinically screened for anti-stroke potential. Hereunder are listed the names of few promising herbal drugs which have been screened for their anti-stroke potential:

Bacopa monnieri	Prakash and Sirsi, 1962; Chowdhuri *et al.*, 2002
Centella asiatica	Brinkhaus *et al.*, 2000; Biswas and Mukherjee, 2003; Guo *et al.*, 2004; Gnanapragasam *et al.*, 2004
Curcumin	Thiyagarajan and Sharma, 2004; Shukla *et al.*, 2008
Ginseng	Bae *et al.*, 2004; Bu *et al.*, 2005; Son *et al.*, 2009
Nardoyscht jatamansi	Tripathi *et al.*, 1996; Salim *et al.*, 2003
Radix Salviae Miltiorrhizae	Kuang *et al.*, 1996; Tang *et al.*, 2002
Shengmai san	Xuejiang *et al.*, 1999; Ichikawa *et al.*, 2003; Wang *et al.*, 2005
Tetrandrine	Sun and Liu, 1995; Liu *et al.*, 2001
Tinospora cordifolia	Rawal *et al.*, 2004; Leyon and Kuttan, 2004
Withania somnifera	Dhuley, 1998; Chaudhary *et al.*, 2003; Gupta *et al.*, 2004b; Mohanty *et al.*, 2004
Vineatrol/ trans-resveratrol	Yavuz *et al.*, 1997; Billard, 2002; Yousuf *et al.*, 2009

Ginsberg, (2008) has made an attempt to collect information regarding the clinical trials made so far using different approaches to study the antistroke potential of synthetic/natural drug candidate molecules. The clinical trials reviewed by them are listed in Fig. 2.1.

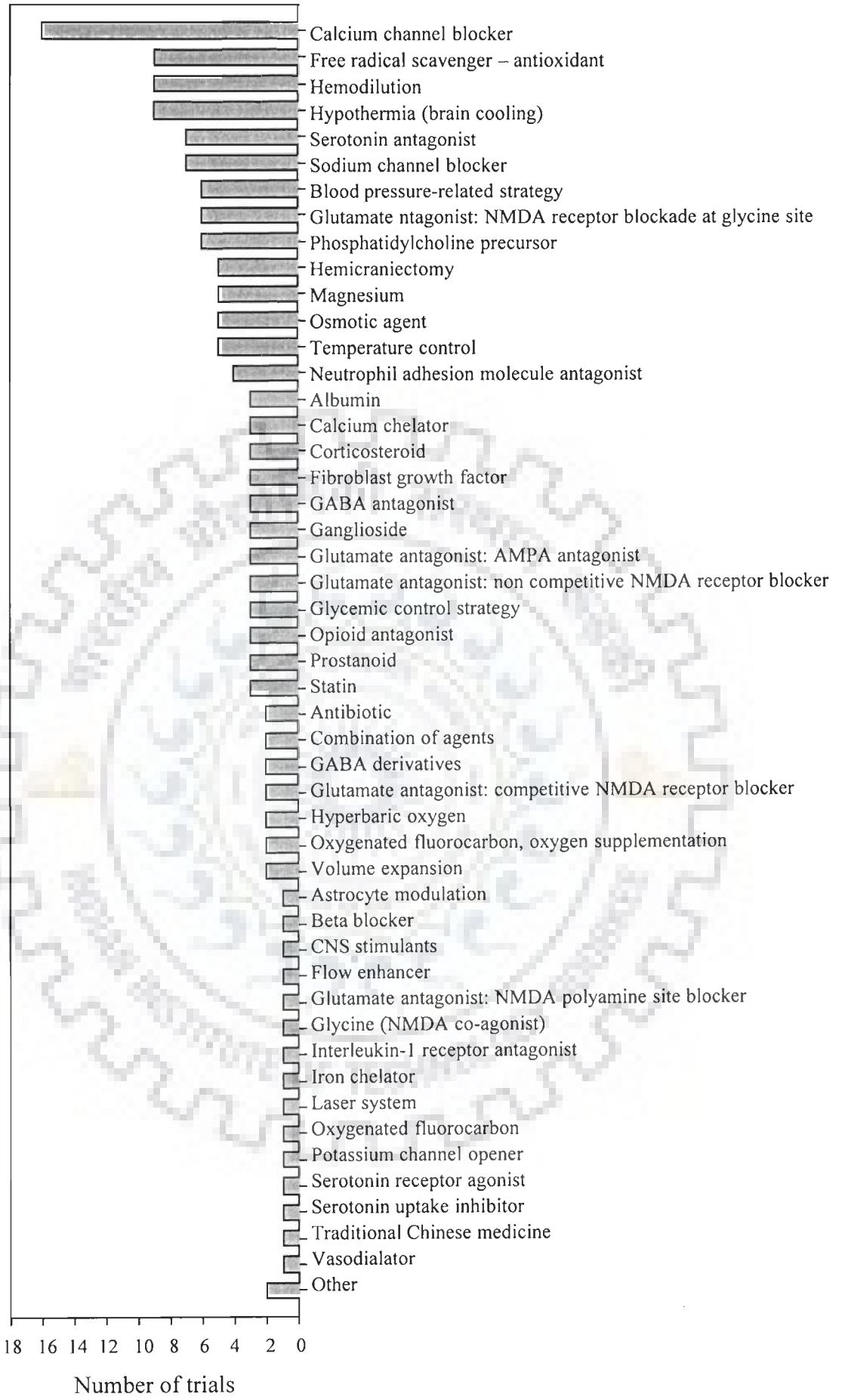


Fig. 2.1 Clinical trials reviewed by Ginsberg, (2008)

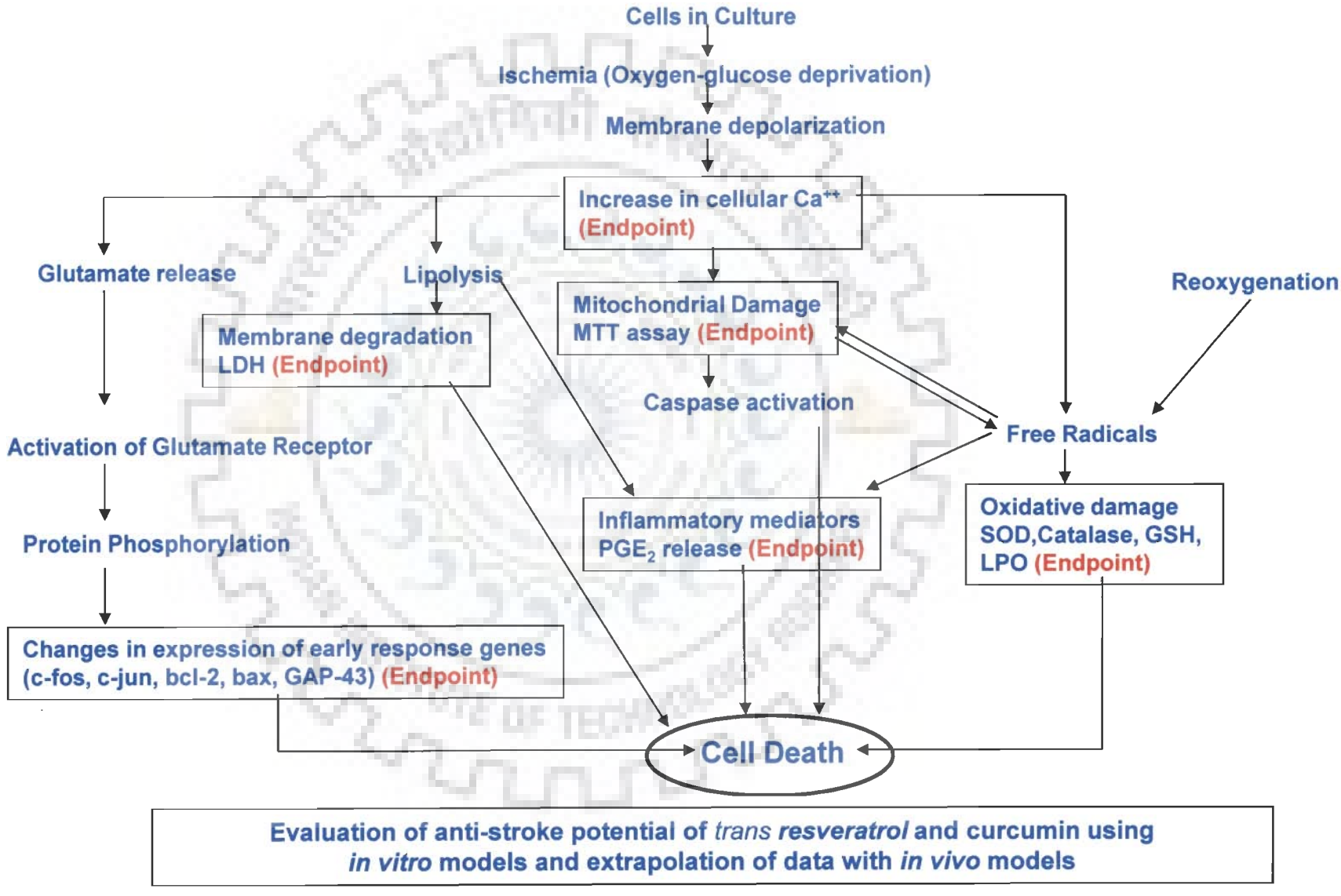


Fig. 3.1. Flow chart of possible correlation between different endpoints, to study the OGD induced changes and known to involve in the pathophysiology of ischemic stroke.

iv. Restoration/regeneration of neurons using stem cell transplantation

Experimental studies have been initiated to use the development and plasticity potential of human umbilical cord blood stem cells to convert them into specific neurons and use them as a tool to restore/regenerate the normal function of brain in stroke patients (Andres *et al.,* 2008). The studies are in primitive stages and encouraging results have been obtained so far from experimental gerbil (Schmidt and Reymann, 2002; Ishibashi *et al.,* 2004), mice (Oyamada *et al.,* 2008) and rat models (Takahashi *et al.,* 2008; Pavlichenko *et al.,* 2008). Many other studies are in progress, the results of which are awaited (Andres *et al.,* 2008). Also, translational research guidelines are being adopted by academic Institutes, industry, National Institutes of Health (NIH) and Food and Drug Administration (FDA) to provide the basis for cell therapy in stroke (Borlongan, 2009).

Stroke research: Indian scenario

In ischemic stroke, free radicals generated massively at the time of reperfusion are the major contributors to neuronal injury (Srivastava *et al.,* 2009). Free radicals are highly reactive species that promote damage to lipid, DNA, carbohydrates and proteins. They also contribute to brain edema, movement of white cells into the ischemic zone (leukocytosis), alteration of blood flow and break down of blood brain barrier. Consistent with these facts, various free radical scavengers have been found to have protective effect against stroke. Antioxidants like resveratrol, melatonin, adenosine (Sinha *et al.,* 2001a; 2001b; Gupta *et al.,* 2002a), trolox (Gupta and Sharma, 2006; Sharma and Kaundal, 2007), alpha-tocopherol (Chaudhary *et al.,* 2003a) were found to have neuroprotective effect against neuronal injury induced by middle cerebral artery occlusion in rats. Combination of melatonin (an antioxidant) and meloxicam (a COX-2 inhibitor) showed enhanced protection in middle cerebral artery occlusion induced acute ischemic stroke in rats (Gupta *et al.,* 2002b). Herbal drugs like *trans* resveratrol (Tsai *et al.,* 2007), *Withania somnifera* (Chaudhary *et al.,* 2003b), *Acorus calamus* (Shukla *et al.,* 2006) and curcumin (Thiyagarajan and Sharma, 2004; Daya *et al.*, 2008; Shukla *et al.,* 2008) have been found to be neuroprotective through their antioxidant activity in rat MCAo model of ischemic stroke. Alpha lipoic acid, a universal antioxidant showed protection against reperfusion injury following cerebral ischemia and reduced the mortality rate in the experimental animal group (Panigrahi *et al.,* 1996). Studies have shown that endothelin antagonist (TAK-044) has

neuroprotective potential in cerebral stroke in both *in vitro* and *in vivo* models (Briyal *et al.*, 2006, 2007).

In few other mechanistic studies, it has been suggested that mild hypothermia significantly decrease the levels of damage following cerebral ischemia (Yanamoto *et al.*, 1996; Kollmar *et al.*, 2002; Rastogi *et al.*, 2006). Babu et al (2002) have confirmed the findings of neuroprotective effect of hypothermia by observing enhanced expressions of Bcl-2, an early response marker for the inhibition of apoptosis, in experimental rats. Bcl-2 is known to prevent apoptosis by blocking the translocation of cytochrome-C and subsequent caspase activation. Further, Brahma et al (2009) have also suggested cytochrome-C dependent apoptosis in global ischemia in gerbils. Curcuma oil, an antioxidant isolated from the rhizomes of *Curcuma longa*, inhibited apoptosis and MCAo induced damages in rat brains (Dohare *et al.*, 2008; Rathore *et al.*, 2008).

Poly (ADP ribose) polymerase (PARP) is a DNA-binding protein that uses nicotinamide adenine dinucleotide (NAD) as a substrate. It protects DNA integrity and facilitates the repair of DNA strand breaks. Over activation of PARP causes massive NAD and ATP consumption resulting in cell death before DNA repair takes place. Further depletion of energy stores under already ischemic conditions can lead to necrosis and apoptosis of neurons. PARP inhibitors have been shown to have protective effect in both *in vitro* and *in vivo* models of ischemic reperfusion injury (Gupta *et al.*, 2004a; Kaundal *et al.*, 2006). Kabra *et al.* (2004) has also confirmed the role of PARP in neuronal cell death by demonstrating the neuroprotective effect of 4-amino-1, 8-napthalimide (4-ANI), a PARP inhibitor in middle cerebral artery occlusion-induced focal cerebral ischemia in rats.

Epidemiological studies carried out in Indian population have shown no adverse metabolic effect of short-term antiplatelet therapy in patients of ischemic stroke. Instead, a rise in serum HDL was observed that may contribute to the cerebral protective effect (Gaur *et al.*, 1993). In randomized controlled double blind clinical trial, nimodipine was found to be beneficial in the recovery of acute cerebral ischemia patients (Nag *et al.*, 1998). Kumar *et al.* (2001) demonstrated the role of stroke in the bone deterioration in the patients suffering from complex regional pain syndrome type-1. Studies of Dhanuka *et al.* (2001) are suggestive that early onset seizures after stroke are more common, did not affect outcome of prophylaxis and did not recur even when

not treated with anti-epileptic drugs whereas late onset of seizures was found to be less common and associated with more recurrences.

***In vitro* models of cerebral stroke**

Experimental models of cerebral ischemia represent an important contribution to both our understanding of human stroke and the development of new therapies. Since last 25 years, rodent models have been established as standard experimental models in all spheres of biomedical research. Subsequently, with technological advancement and to avoid the ethical dubious, cells isolated from different organs as well as immortal cell lines also got popularized as first line of experimental tools to understand the intra and extracellular biological reactions. Thus, in order to understand the mechanisms involved in cerebral stroke and to screen new drug candidates for their therapeutic potential, attempts are continuously being made to develop various *in vivo* and *in vitro* models of cerebral stroke.

In last couple of decades, *in vitro* model systems got popularized to understand the target specific mechanistic studies. In these *in vitro* systems, there is no interference of indigenous factors and they are also helpful in reducing the ethical dubious of animal use in biomedical research. Few initiatives have already been taken using cell lines viz., PC12 cells (Kang *et al.,* 2006; Ito *et al.,* 2006; Singh *et al.,* 2009); immortal human neuroblasts cell lines like Neuro-2a, SH-SY5Y (Miglio *et al.,* 2004; Fordel *et al.,* 2007; Zhu *et al.,* 2007; Chang *et al.,* 2008), U-87 (Santra *et al.,* 2006), C-6 glioma cells (Shi *et al.,* 2006), primary cultures of neuronal cells (Malagelada *et al.,* 2005; Briyal *et al.,* 2006; Luo et al., 2006; Zhu *et al.,* 2007), mixed cultures of neuronal and glial cells (Canas et al., 2006) and rat brain slices (Jung *et al.,* 2008; Konrath *et al.,* 2008; Zhang *et al.,* 2008) towards development of *in vitro* models to understand the mechanisms involved in the pathophysiology of cerebral stroke. Most of the *in vitro* studies are broadly focused to understand the factors involved and events happening during the reoxygenation period since prompt reperfusion of ischemic brain tissue is critical for restoring normal function. Attempts were made to study whether cultured PC12 cells could be a model of choice to understand the mechanism(s) involved in ischemic cerebral stroke and whether the data would be extrapolative to rat MCAo model of cerebral stroke (Tabakman *et al.,* 2002). The PC12 cells-OGD *in vitro* model was used to study its responsiveness for endpoints involved in the pathophysiology of ischemic stroke following exposure of known

neuroprotectants viz., carnosine and homocarnosine (Tabakman *et al.,* 2002), aminoguanidine (Jiang *et al.,* 2005), scutellarin (Xu *et al.,* 2007), naloxone (Huang *et al.,* 2008) and catalpol (Wang *et al.,* 2008).

Kaushal and Schlichter (2008) have demonstrated the utility of three-stage *in vitro* experimental paradigm of the stroke penumbra. In the first stage, neuron and astrocyte co-cultures were placed under oxygen and glucose deprivation (OGD) for various times, simulating the stroke core. In the second stage, these OGD-stressed co-cultures (OGD-SCs) were co-incubated for 24 h with microglia allowing soluble factors released from OGD-SCs to activate the microglia. In the third and final stage, microglia were washed and incubated with naive neuron/astrocyte co-cultures for 48 h. By using these integrated approaches, they have developed an *in vitro* model to study the key signaling factors that may contribute to cellular deterioration within the stroke penumbra.

Brain slices (Balestrino *et al.,* 2002; Rytter *et al.,* 2003; Noraberg *et al.,* 2005; Windelborn and Lipton, 2008; Cui *et al.,* 2009) and neuronal cultures (Arias *et al.,* 1999; Gee *et al.,* 2006; Ge *et al.,* 2007; Yang *et al.,* 2007; Antonelli *et al.,* 2008) have been found to have utility in screening drug molecules having anti-stroke potential including glutamate receptor antagonists since extracellular concentrations of glutamate receptors increase rapidly following ischemic insult (Arias *et al.,* 1999; Zimmer *et al.,* 2000).

Many studies have been carried out showing added advantages of use of *in vitro* brain slices over the other preparations of brain including the whole brain (Sick and Somjen, 1998; Lipton, 1999; Rytter *et al.,* 2003). Hippocampal slices were suggested as better test models to screen the test compounds due to controlled environmental conditions, no extracellular fluid and direct availability of receptors to the test compound during both ischemia and re-perfusion period (Boscia *et al.,* 2006; Cui *et al.,* 2009). Unlike neuronal cultures, brain slices largely retain both the cytoarchitecture and the electrophysiological characteristics of the intact hippocampus. Moreover, the intrinsic cytoarchitecture of the hippocampal slice allows generation of electrophysiological field potentials that are quite similar to those of the intact animals and provide an immediate index of neuronal viability (Arias *et al.,* 1999; Kristensen *et al.,* 2001; Canals *et al.,* 2008). This readily measurable physiological parameter stands in contrast to the more labor-intensive indices of viability inherent in other

preparations e.g., histological appearance, LDH release and propidium iodide fluorescence (Arias *et al.,* 1999). Thus, acute hippocampal slice preparation has been used extensively to address the basic issues surrounding the induction of excitotoxic and ischemic damage in nervous tissue as well as the neuroprotection of tissue subjected to ischemic insults (Canals *et al.,* 2008).

Primary neuronal/glial cultures from cortex (Ge *et al.,* 2007; Yang *et al.,* 2007; Antonelli *et al.,* 2008), hippocampus (Montero *et al.,* 2007; Windelborn and Lipton *et al.,* 2008; Cui *et al.,* 2009), cerebellum (Zheng and Zuo, 2003; Isaev *et al.,* 2004; Tastekin *et al.,* 2005) and hypothalamus (Miao *et al.,* 2007) of embryo or perinatal rats and mice have also been suggested to have utility for study of anoxic or ischemic damage. Of these, the use of organotypic hippocampal slice cultures from perinatal rats is more common. They promise to become more valuable models, particularly as they show delayed cell death (Rytter *et al.,* 2003; Cui *et al.,* 2009). But they were found to have selective vulnerability of CA1 versus CA3, if the *in vitro* ischemia occurs for relatively shorter period i.e. 30 min (Strasser and Fischer, 1995; Barth *et al.,* 1996; Kim *et al.,* 2008) while the cell death becomes almost equal in all hippocampal regions when ischemic insult is for 60 min (Strasser and Fischer, 1995; Reyes *et al.,* 1998; Gee *et al.,* 2006). Damage usually develops 8-24 h following 30-60 min of ischemic insult under *in vitro* conditions and is generally monitored as a gross increase in membrane permeability to dyes or protein, in particular leak of LDH (Myers *et al.,* 1995; Antonelli *et al.,* 2008) or nonexclusion of trypan blue or propidium iodide (Strasser and Fischer, 1995; Laake *et al.,* 1999; Cui *et al.,* 2009). Further, apoptotic and necrotic changes have also reported (Gwag *et al.,* 1995; Siesjo, 2008).

Although 30-60 min deprivation is not long for focal insults, the degree of oxygen and glucose deprivation that cultures are exposed to is similar to that in global ischemia, where 5-10 min at 36°C produced profound delayed damage. There are several possible reasons for the difference. In the widely used model developed by Goldberg and Choi, (1993), ATP does not fall nearly as much as it does in *in vivo* models and probably, the release of glutamate is delayed. The small fall in ATP would explain the requirement for the prolonged insult because it approximates that in the focal ischemic penumbra (Goldberg and Choi, 1993; Hochachka *et al.,* 1996; Siesjo, 2008). Although the alterations in expression (mRNA and protein) are significantly higher in neuronal cultures, they have less anatomical relationship with different brain

parts (Zhou *et al.,* 1995). These limitations are not there with organotypic cultures (Finley *et al.,* 2004).

In vitro systems are considered as the best tools to provide clues to understand the mechanism(s) involved in stroke (Cho *et al.,* 2004; Bonde *et al.,* 2005; Boscia *et al.,* 2006; Cui *et al.,* 2009). However, due to a number of reasons like absence of blood flow and blood vessels in cultures, it is not possible to extrapolate the results to the *in vivo* situations. Thus, to overcome these problems, brain slices are being used (Arias *et al.,* 1999; Kristensen *et al.,* 2001). However, brain slices are in a compromised metabolic state, with low ATP values and elevated aerobic glycolysis (Kristensen *et al.,* 2001; Siesjo, 2008) and are hypersensitive to ischemic insults. They show different morphologies from *in vivo* tissue and the preparation of the slice itself induces transcription of some stress-related mRNA and immediate early response genes as well as accumulation of Fos and Jun immunoreactivity (Zornow, 1995; Gerlach *et al.,* 2002).

To improve the understanding of neuronal cell swelling in cerebral ischemia, cell volume regulation, viability, intracellular electrolytes and lactate production were studied using neuroblastoma cells Neuro-2a as an *in vitro* model (Mackert *et al.,* 1996; Cheung and Hart, 2008; Hsieh *et al.,* 2008). The volume regulatory capacity of Neuro-2a cells was assessed after incubation in hypo- and hypertonic media. Anoxia was studied alone and together with inhibition of glycolysis by iodoacetate (Andres *et al.,* 1996; Mackert *et al.,* 1996). It has also been reported that the final cell volume after regulation depends on the tonicity of the medium and remain above control. There was no regulatory volume increase after cell shrinking in hypertonic media. Despite the severe isotonic incubation, viability decreased only slightly without reaching statistical significance (Mackert *et al.,* 1996; Kuhrt *et al.,* 2008). In contrast to *in vivo* conditions, anoxia for 90 min with or without iodoacetate for additional inhibition of anaerobic energy metabolism neither caused neuronal cell swelling nor a decrease of viability. Reoxygenation after the anoxic period also did not induce volume and viability changes. These results confirm that neuronal cells are able to adequately regulate cell volume in response to hypo-osmotic stress (Mackert *et al.,* 1996; Kuhrt *et al.,* 2008). On the other hand, maintenance of a normal cell size during complete energy deprivation suggests strongly that energy failure *per se* does not suffice to induce neuronal swelling. Cell swelling in cerebral ischemia *in vivo* thus appears to be a

secondary phenomenon due to mediator mechanisms such as tissue acidosis or elevated extracellular glutamate levels (Mackert *et al.,* 1996; Hsieh *et al.,* 2008).

Cell volume homeostasis and the ability to regulate cell volume after swelling from pathophysiological causes is one of the fundamental properties of most cell types (Macknight, 1988, Chamberlin and Strange, 1989; Mackert *et al.,* 1996; Mongin al, 2007). The characteristic feature of cytotoxic brain edema, however, is a pathologic increase of the volume of brain cells; typical especially for cerebral ischemia, brain trauma and metabolic disorders where swelling of nerve and glial cells is a predominant feature (Baethmann, 1978; Mackert *et al.,* 1996). The complexity of the *in vivo* mechanisms of cytotoxic brain edema has been reduced by using variety of *in vitro* models viz., C6 glioma cells (Kempski *et al.,* 1988), primary cultures of astrocytes (Kempski *et al.,* 1991) and Neuro-2a cells (Mackert *et al.,* 1996), which allows observance of central nervous system cells in isolation under strictly defined extracellular conditions. Moreover, the *in vitro* approaches permit a better assessment of cell swelling and viability, parameters which are quantified by flow cytometry with high precision. Using this model with glial cells, substantial swelling-inducing properties have been demonstrated before: elevated levels of H^+ (Kempski *et al.,* 1988; Staub *et al.,* 1990), K^+ (Kempski *et al.,* 1991), glutamate (Schneider *et al.,* 1992) and free fatty acids (Staub *et al.,* 1995) cause swelling of glial cells, whereas up to two hours of anoxia do not disturb cell volume homeostasis (Kempski *et al.,* 1987). Studies with Neuro-2a cells proved that acidosis propagates rapid swelling that is osmotically dependent on Na^+ influx after activation of Na^+/H^+ exchange (Staub *et al.,* 1991, 1993). These advantages of the *in vitro* model allow assessment of basal aspects of neuronal volume control such as regulatory volume decrease and increase after the induction of substantial cell volume changes from abrupt variations of the tonicity of the incubation medium (Hoffmann and Simonsen, 1989).

The *in vitro* experimental model developed so far allows determining dynamically and accurately changes in cell volume, viability, intracellular ion concentration and metabolite production under strictly defined conditions (Okada *et al.,* 2006; Szydlowska *et al.,* 2006; Mongin, 2007; Hsieh *et al.,* 2008; Hirrlinger *et al.,* 2008; Kuhrt *et al.,* 2008; Siesjo, 2008). Uncontrolled changes in the intra and extracellular homeostasis occurring *in vivo* in brain tissue during experimental procedures can be ruled out since temperature, ion concentration and osmolality are

generally controlled and maintained during the use of these *in vitro* models. It has been observed that alterations in the medium during experiments by cellular release of lactate, K^+ ions or excitatory amino acids, especially during energy deprivation, are negligible because of the large extracellular to intracellular volume ratio (Leis *et al.*, 2005; Abdullaev *et al.*, 2006; Hsieh *et al.*, 2008; Hirrlinger *et al.*, 2008; Kuhrt *et al.*, 2008).

Materials and Methods

3. MATERIALS AND METHODS

Reagents and consumables

All the specified chemicals, reagents, diagnostic kits and antibodies were purchased from Sigma St. Louis, MO, USA, unless otherwise stated. Culture medium (Dulbecco's Modified Eagle's Medium), fetal bovine serum and horse serum were purchased from Gibco BRL, USA. Antibiotic-antimycotic solution was procured from Invitrogen, Life Technologies, USA. Culture wares and other plastic consumables used in the study were procured commercially from Nunc, Denmark. Milli Q water (double distilled deionized water) was used in all the experiments.

Cell culture

PC12 cells, a rat pheochromocytoma cell line used in the study was originally procured from National Centre for Cell Sciences (NCCS), Pune, India and since then it has been maintained at In Vitro Toxicology Laboratory, Indian Institute of Toxicology Research, Lucknow, India, as per the standard protocols. In brief, the cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2.5% fetal bovine serum (FBS), 15% horse serum, 0.2% sodium bicarbonate and antibiotic-antimycotic solution (10X, 1% of medium). The cells were grown in 5% CO_2-95% atmosphere, in high humidity at 37°C. Medium was changed twice weekly and the cultures were split at a ratio of 1:6 once a week. Each batch of cells was assessed for cell viability by trypan blue dye exclusion test, prior to being selected for experiment and batches showing more than 95% cell viability were used in the study. Cells of passage number 18-25 were used in the study.

Marker identification

Prior to use in the experiments, the genotypic and phenotypic integrity of PC12 cells was assessed by expression analysis for neuronal specific genes. In brief, following the declustering, adequate number of cells were seeded in poly-L-lysine (PLL) precoated multi-well culture plates and allowed to adhere for 24 h at 37°C in 5% CO_2-95% atmosphere under high humid conditions. Since, PC12 cells are loosely adhered cells and tend to grow in small clusters, a pre-coating of PLL facilitates tight adherence of cells to the plastic surface. Following adherence, medium was aspirated and replaced with fresh medium supplemented with nerve growth factor (NGF, 50 ng/ml). NGF containing medium was replaced at every alternate day till the complete

maturation i.e. 21 days. Cells were observed and photographed at regular intervals to study the morphological differentiation using inverted phase contrast microscope (Leica DMIL, Germany). NGF induced differentiating PC12 cells were analyzed for the expression of neuronal specific gene proteins viz., nestin, β-tubulin, neurofilament L (NF-L), neurofilament M (NF-M), neuritin 5 and dopamine DA-D_2 receptor all through the differentiation. Translational changes were quantified at protein level by both western blotting and immunocytochemistry using specific monoclonal antibodies.

Oxygen-glucose deprivation (OGD)

Adequate numbers of cells showing viability more than 95% were collected by centrifugation at 600 rpm for 6 min. Following two washings with sterile PBS (pH 7.4), the cells were re-suspended in glucose free DMEM supplemented with horse serum, fetal bovine serum, sodium bicarbonate and antibiotic-antimycotic solution as in the standard cultures and allowed to grow in an atmosphere 5% CO_2-94% N_2 -1% O_2 under high humid conditions at 37°C for 1-8 h. Immediately following the OGD insult for 1-8 h at an interval of 1 h, fresh medium containing a range of glucose concentrations was added in the cultures in a way that the final concentration of the glucose in the medium was in increasing order i.e., 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg/ml. To make the conditions more ideal, glucose free medium used during OGD insult was not aspirated from the cultures. Thus, the secretions collecting in the medium due to OGD insult might contribute to the detrimental effects of reoxygenation process. Cultures growing in medium with different glucose concentrations were then subjected to reoxygenation for either of 6, 12, 18, 24, 36, 48, 72 and 96 h at 37°C in 5% CO_2-95% atmosphere under high humidity.

Cell viability by trypan blue dye exclusion test: The test was conducted to study the cell viability by assessing the loss of membrane integrity following the method of Pant *et al.* (2001). In brief, the cells (1×10^4/well) were seeded in 96-well culture plates (Nunc, Denmark) and allowed to grow for 24 h in 5% CO_2 - 95% atmosphere at 37°C under high humid conditions. The medium was then replaced with glucose free medium and cells were allowed for OGD insult as described earlier. Following the OGD insult, cells were subjected to assess the loss of cell viability. Immediate after the completion of respective OGD insult, cell suspensions were aspirated, centrifuged at 600 rpm for 5 min, washed twice with sterile PBS (pH 7.4) and re-suspended in small amount of PBS. The cell suspension was then mixed with trypan blue dye (0.4%

solution) at a ratio of 1:5 (dye: cell suspension) and placed in heamocytometer. The counting for live (unstained) and dead (blue stained) cells was made at 100X magnification in phase-contrast inverted microscope (Leica DMIL DMIL, Germany). Parallel sets were also run under identical conditions without OGD insult and served as control. Percent cell viability was calculated by using the following formula.

% Cell viability = [(No. of unstained cells) / (No. of unstained + stained cells)] × 100

MTT assay: It depicts mitochondrial dehydrogenase activity of live cells to convert the pale yellow 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) salt into a purple/violet formazon. MTT assay provides an induction in the activity and integrity of mitochondria against any physical and pathophysiological stress and is usually interpreted as a measure of percent cell viability. The assay was performed following the protocol of Pandey *et al.* (2006) with required modifications. Cells (1×10^4 per well) were seeded in poly-L-lysine pre-coated 96-well culture plates and allowed to adhere properly for 24 h at 37°C in CO_2 incubator (5% CO_2-95% atmosphere at high humidity). The medium was then replaced with glucose-free medium and cells were subjected to OGD insult of 6 h followed by reoxygenation of 6-96 h. The protocols for OGD insult and reoxygenation were followed as described earlier. Tetrazolium salt (5 mg/ml of stock in PBS) was added (10 µl/well containing 100 µl of cell suspension) 5 h prior to the completion of reoxygenation period and cultures were then re-incubated till the end of reoxygenation period. The reaction mixture was carefully aspirated and 200 µl of culture grade dimethyl sulfoxide (DMSO) (Sigma St. Louis, MO, USA) was added to each well. Solubilization of the formazon was ensured by pipetting up and down several times. Culture plates were left to rest at room temperature for ten minutes and then read at 530 nm using multiwell microplate reader (Synergy HT, BioTek, USA). Parallel sets were also run under identical conditions without OGD insult in normoxia condition and served as control. Identical protocol was followed to carry out the MTT assay for all the reoxygenation periods selected in the study i.e., 6, 12, 18, 24, 36, 48, 72 and 96 h.

The data obtained through trypan blue dye exclusion and MTT assays suggested the suitability of OGD of 6 h followed by a reoxygenation period of 24 h in culture medium containing 4-6 mg/ml glucose concentrations as the optimum conditions to create cerebral stroke like situations under *in vitro* environment using

PC12 cells. To further find out the possible effected endpoints and points of therapeutic interventions, studies were carried out using these conditions only. Since, the concentration of glucose in commercially available culture medium is 4.5 mg/ml, which falls within the range of the experimental conditions, no alteration in glucose concentration was made in the medium used for the study. Protocols of endpoints discussed hereafter are given with the understanding that all the experiments were carried out using standardized conditions of OGD and reoxygenation period and compared with normoxia control sets run parallel under identical conditions. The criteria for the selection of endpoints for *in vitro* studies are given in Fig. 3.1. This is a flow chart of possible correlation between different endpoints, which were looked at to study the OGD induced changes and are known to be involved in the pathophysiology of ischemic stroke.

Intracellular calcium levels $[Ca^{2+}]_i$: Alterations in the levels of intracellular calcium in cells of experimental group vs. control were assayed by fluorometric quantification of hydrolysed acetylmethyl ester of quinoline tetracarboxylic acid (Quin-2AM) (Komulainen and Bondy, 1987). Cells (1×10^6) were incubated in 3.0 ml assay buffer (120 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 6 mM glucose, 6 mM $CaCl_2$, 25 mM Hepes, pH 7.4) along with Quin-2AM (25 mM final concentration) at 37°C for 45 min in the dark. The assay mixtures were then centrifuged at 1000 rpm for 10 min and the pellets were washed three times to remove excess of Quin-2AM. Finally pellets were suspended in 1.0 ml assay buffer. Fluorescence 'R' was measured on spectrofluorimeter (LS 55 Perkin Elmer, UK) using an excitation and emission wavelengths of 330 nm and 492 nm respectively. Fluorescence 'R minimum' (R_{min}) was observed after the addition of 0.1% sodium dodecyl sulphate (SDS) and EGTA (10 mM). Fluorescence 'R maximum' (R_{max}) was determined after adding 7 mM of $CaCl_2$. Intracellular calcium levels were then calculated using the formula:

$$\text{Intracellular } [Ca^{2+}] = Kd\ (R - R_{min}) / (R_{max} - R)$$

where, Kd is the dissociation constant of Quin-2AM-Ca^{2+} complex and is equivalent to 115 nm. The results are expressed in terms of n mol of calcium.

Mitochondrial membrane potential: On the completion of reoxygenation period of 24 h, cells were collected by centrifugation at 600 rpm for 6 min and re-suspended in 0.1 M PBS (pH 7.4). Single cell suspension was made by repeated pipetting. The cells

were then incubated with Rhodamin123 (Rh^{123}) (Sigma St. Louis, MO, USA) at a final concentration of 1 μM for 1 h at 37°C in dark. Subsequently they were washed twice to remove excess dye and re-suspended again in PBS. The fluorescence was then measured on flowcytomerter (Becton Dickinson LSR2, San Jose, USA) at the excitation and emission wavelength λ_{exc} = 488 nm, λ_{em} = 525 nm respectively, for at least 10000 events per sample. The data collected was analyzed using data acquisition program "Cell Quest 3.3". The specificity of Rh^{123} to translocate into the active mitochondria was verified using PC12 cells pre-incubated for 10 min with protonophore CCCP (50 μM), which depolarizes the mitochondrial membrane. Alterations in fluorescence signal in the absence or presence of the protonophore were then scored.

Membrane degradation by LDH release assay: Lactate dehydrogenase (LDH) release assay is a method to measure the membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The assay was carried out using commercially available LDH assay kit for *in vitro* cytotoxicity evaluation (TOX-7, Sigma St. Louis, MO, USA). The assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD ($NADH^+$) is utilized in the stochiometric conversion of a tetrazolium dye. The resulting colored compound is measured using multiwell plate reader (Synergy HT, BioTek, USA) at wavelengths 490 and 690 nm. In brief, after the ischemic insult and desired reoxygenation period, the cells were processed for LDH release assay similar to MTT assay. Culture plates were removed from CO_2 incubator as per the experimental schedule and centrifuged at 250 × g for 4 min. The supernatant of each well was transferred to a fresh flat bottom 96-well culture plate and processed further for enzymatic analysis as per the manufacturer's instructions.

Prostaglandin E_2 (PGE_2): Adequate number of cells were seeded in culture flasks and allowed to grow under normoxia for 24 h. The medium was then aspirated and batches of cells were processed for OGD insult and reoxygenation as described earlier. On the completion of reoxygenation period, culture medium of the respective flasks was collected after centrifugation at 1000 rpm for 10 min and processed for the estimation of PGE_2 using commercially available kit (PGE_2 assay kit, Catalog No. 514010, Cayman Chemicals, USA) following the manufacturer's protocol. At least two wells were designated for Blank (Blk), Total activity (TA), Non-specific binding

(NSB), Maximum binding (B_0), Standards (Std) and Samples (S) in the 96-well culture plate. 100 µl of EIA buffer was added to NSB wells and 50 µl to B_0 wells. Following it, 50 µl of standards were added starting from the lowest concentration that was prepared separately in cleaned glass tubes. Samples (50 µl) were added in the respective wells. Prostaglandin E_2 AChE tracer (50 µl) was added to each well except the TA and Blk wells. Following it 50 µl of PGE_2 monoclonal antibody was added to each well except the TA, NSB and Blk wells.

Well	EIA buffer	Standard/Sample	Tracer	Antibody
Blk	-	-	-	-
TA	-	-	5 µl (at development step)	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

After the addition of all these reagents, the plates were covered with plastic film and incubated for 18 h at 4°C. Following the completion of the incubation, wells were washed five times with wash buffer followed by addition of freshly prepared Ellman's reagent (200 µl) to each well and 5 µl tracer to the TA wells. The plates were again covered with plastic film and allowed to develop in dark on orbital shaker for 90 min. The plates were read at 410 nm using microplate reader (Synergy HT, BioTek, USA).

Standard curve was plotted by taking % B/B_0 values on Y-axis and concentrations on X-axis. The concentrations of PGE_2 in the samples were determined by identifying the % B/B_0 on the standard curve and the corresponding values on the X-axis.

Lipid peroxidation (LPO): Following OGD insult and adequate reoxygenation period, cells were harvested by centrifugation at 1000 × g for 10 min and processed for the estimation of lipid peroxidation using commercially available kit (Lipid Peroxidation Assay Kit, catalog no. 705002; Cayman Chemicals, USA) as per the manufacturer's protocol. The collected cells were sonicated in HPLC-grade water and lipid hydroperoxides were extracted from the samples into chloroform before

performing the assay. Chloroform-ethanol solvent mixture (450 µl) was added to chloroform extract (500 µl) of each sample. Freshly prepared chromogen (50 µl) was then added to each assay tube and mixed well on a vortex mixture. Tightly caped assay tubes were kept at room temperature for 5 min. Final suspension (300 µl) of assay tubes and standard tubes were then transferred to 96-well plates and read at 500 nm using the microplate reader (Synergy HT, BioTek, USA). Standard curve was plotted using lipid hydroperoxide standard supplied by the manufacturer with kit.

Catalase activity: The experimental setup for catalase activity was similar to the LPO. Activity was measured using commercially available kit for catalase activity (Catalog no. 707002; Cayman Chemicals, USA) following the protocol provided by manufacturer. Following the completion of reoxygenation period, cells were collected by centrifugation at 1000 × g for 10 min at 4°C, sonicated in 1.0 ml cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA) and centrifuged at 10000xg for 15 min at 4°C. The supernatant was collected for assay. Then 100 µl of assay buffer (supplied in the kit), 30 µl of methanol and 20 µl of sample were mixed in the 96-well plate. Reaction was initiated by adding 20 µl of hydrogen peroxide (0.882 M) and incubated in shaker for 20 min at room temperature and stopped by adding 30 µl of potassium hydroxide. Chromogen (30 µl) was added and it was incubated for 10 min followed by addition of potassium periodate (10 µl). The plates were kept at room temperature for 5 min and read at 540 nm using microplate reader (Synergy HT, BioTek, USA). Bovine liver catalase (supplied in the kit) was used as positive control in the assay. Standard curve was plotted using formaldehyde between 0 and 75 µM.

Superoxide dismutase (SOD) activity: The experimental setup for SOD activity was similar to LPO and catalase. The activity was measured using commercially available kit for superoxide dismutase activity (Catalog no. 706002; Cayman Chemicals, USA) following the protocol provided by manufacturer. After completion of reoxygenation period, cells were collected by centrifugation at 1000 × g for 10 min at 4°C and sonicated in Hepes buffer (20 mM, pH 7.2, containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose). It was then centrifuged at 1500 × g for 5 min at 4°C and supernatant was collected for assay. Assay sample (10 µl) was mixed with 200 µl radical detector (supplied in the kit). Reaction was initiated by adding 20 µl xanthine oxidase. The plate was incubated on shaker for 20 min at room temperature and the

absorbance was read at 450 nm using microplate reader (Synergy HT, BioTek, USA). Standard curve was plotted using the SOD standard supplied in the kit.

Glutathione (GSH) content: In a similar setup like LPO, catalase and SOD, the change in glutathione contents was also measured using commercially available kit for glutathione assay (Catalog No. 703002; Cayman Chemicals, USA) following the manufacturer's protocol. After completion of reoxygenation period, cells were collected by centrifugation at 1000 × g for 10 min at 4°C and sonicated in cold MES buffer (50 mM, pH 6.0, containing 1 mM EDTA). It was then centrifuged at 10000 × g for 15 min at 4°C and supernatant was collected and deproteinated for the assay. 50 µl of deproteinated sample was put in 96-well plate and 150 µl freshly prepared assay cocktail was added. Absorbance was read at 405 nm using microplate reader (Synergy HT, BioTek, USA) at 5 min intervals for 30 min. Standard curve was plotted using the oxidized glutathione standard supplied in the kit.

Reactive oxygenation species generation (Flowcytometeric analysis): Experimental setup for reactive oxygenation species (ROS) generation was similar to other endpoints of oxidative stress studied. On completion of reoxygenation period of 24 h, cells were collected by centrifugation at 600 rpm for 6 min and re-suspended in 0.1 M PBS. Single cell suspension was ensured by repeated pipetting. The cells were then incubated with 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA; Sigma Aldrich, USA) dye as fluorescence agent at a final concentration of 5 mM for 30 min at 37°C. As non-specific esterases are known to cleave the acetoxymethyl group of DCFH-DA and resultant to that a non-fluorescent charged molecule is formed which cannot cross the cell membrane. The intracellular ROS oxidizes these charged non-fluorescent molecules to dichlorofluorescein (DCF) fluorescent product. They were then washed twice to remove excess dye and re-suspended in PBS. Fluorescence was measured on flowcytomerter (Becton Dickinson LSR2, San Jose, USA) at the excitation and emission wavelength λ_{exc} = 488 nm and λ_{em} = 525 nm respectively, for at least 10000 events per sample. The data so collected was analyzed using data acquisition program Cell Quest 3.3.

Reactive oxygen species generation (Fluorescent microscopy): The fluorescence measurement was done by intracellular quantification using fluorescence microscope. The assay was carried out following the protocol of Halliwell and White (2004). In

brief, cells ($5x10^4$ per well) were seeded in Poly L- Lysine pre-coated tissue culture chamber slide and allowed for adherence. On completion of reoxygenation period of 24 h, cells were washed twice with PBS and incubated for 30 min in dark in incomplete culture medium containing DCFH-DA (20 µM). Then, the slides were washed twice with PBS and mounted for microscopic analysis. Intracellular fluorescence was measured using upright fluorescence microscope by grabbing the images (Nikon Eclipse 80i equipped with Nikon DS-Ri1 12.7 megapixel camera). Quantification of fluorescence was done using image analysis software Leica Qwin 500 and data expressed in percent of unexposed control.

Nitric oxide (NO) generation: In an identical setup, experiments were carried out to estimate the changes in generation of NO using commercially available kit (Nitric Oxide Colorimetric Assay Kit, Catalog No. K262-200; Bio-Vision, USA) following the protocol provided by the manufacturer. After the completion of reoxygenation period, extracellular solution was collected separately from each well. The collected solution (80 µl) was mixed with 10 µl enzyme cofactor and 10 µl nitrate reductase mixture and the plate was incubated for 2 h at room temperature. 50 µl Griess reagent R1 was added to it following incubation and the color was allowed to develop for 10 min. Absorbance was read at 540 nm using microplate reader (Synergy HT, BioTek, USA). Absorbance of the blank, where only 200 µl assay buffer was added, was subtracted from the absorbance recorded for the samples and standards.

Dopamine (DA-D_2) receptor analysis: On completion of reoxygenation period of 24 h, the cells were collected by centrifugation at 1000 rpm for 10 min at 4°C. The cell pellets were then suspended in 5 mM Tris HCl buffer (pH 7.4) and sonicated. The sonicated cell suspension was then centrifuged at 20,000 rpm for 20 min at 4°C. The membrane pellets were suspended in 40 mM Tris HCl buffer (pH 7.4) and were stored at -80°C till assay. Protein estimation of each sample was done following the method of Lowry *et al.* (1951) using bovine serum albumin as a reference standard.

Dopamine (DA-D_2) receptor modulation by OGD insult and reoxygenation was studied in cultured PC12 cells following the protocol of Seth *et al.*, (2002a) with desired modifications. Specific radioligand ^{3}H-Spiperone (15 Ci/mmole, 1×10^{-9} M) was used for the analysis of DA-D_2 receptors in PC12 cells. Haloperidol (1×10^{-6} M) was used as positive competitor. Radioligand receptor binding assay was carried out

using Robotic Arm Liquid Handling High Throughput System (Multi Probe II EX, Packard, USA)(Photograph 3.1). Multiwell plates (Millipore MSFBN6B, 96-well) were arranged on the deck and mapped with the WINPREP software. Sequential addition of the assay components was also programmed. Adequate amount of cell membrane and specific radioligand (^{3}H-Spiperone) was added in each well and the final volume was made up to 250 µl by adding Tris buffer (40 mM, pH 7.4). Binding assay was carried out in triplicate in each case and specific competitor was run to titrate the assay system. The reaction mixture in the plate was incubated for 15 min at 37°C followed by filtration of the contents over vacuum manifold. Plates were washed twice with buffer, filtered over vacuum and dried. Scintillation cocktail (Microscint O, Packard, USA) 70 µl was added to each well and plates were left overnight. Counting as counts per minute (cpm) was carried out using plate counter (Top Count-NXT, Packard, USA). Binding in the absence of competitor is considered to be the total binding, whereas, in presence of competitor binding, it is regarded as non-specific binding. Finally, percent change in the specific binding following OGD insult to PC12 cells was calculated.

Transcriptional studies

OGD and reoxygenation induced alteration and curcumin and *trans* resveratrol mediated restoration in the mRNA expression of marker genes known to be associated with ischemic cerebral stroke were carried out using semi-quantitative reverse transcriptase-PCR method. Genes showing significant up or down regulation in semi-quantitative PCR were further studied by real time-quantitative PCR (RT-PCR^q).

RNA isolation and RT-PCR: Total RNA was isolated using triZOL reagent (Gibco, BRL) as per the manufacturer's protocol. RNA (1 µg) was used further for RT-PCR in a final volume of 20 µl. The first strand synthesis was done under the conditions of: 50 mM tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM spermidine, 0.25 mM of each dNTP, 12.5 pmol each of oligo (dT) and random hexamer primers, and 5-8 U of avian myeloblastosis virus (AMV) reverse transcriptase (Promega, USA). After 1 h incubation at 42°C, the reaction mixture was diluted to 100 µl with sterilized deionized water and stored at -80°C. Subsequently 1-2 µl was used for PCR.

Photograph 3.1: Robotic Arm Liquid Handling High Throughput System

Semi-quantitative PCR analysis: PCR was carried out in a volume of 23 µl containing Tris-HCl (10 mM, pH 9.0), KCl (50 mM), $MgCl_2$ (2 mM), 0.2 mM each of dNTP and appropriate primers (0.8 uM). After an initial denaturation for 4 min at 95°C, 2 µl of *Taq* polymerase (0.5 U/µl) was added and 20-40 cycles were performed in thermocycler, each consisting of 30 sec of denanaturation at 95°C, 30 sec of primer annealing at 52-65°C, depending on the primer pair and 1 min of elongation at 72°C. After a terminal extension for 5 min at 72°C, amplification products were stored at 4°C of which 5-10 µl was used for gel electrophoresis on 1.5% agarose gel. Gels were analyzed for up or down regulation of mRNA expression using gel documentation system (Alpha Innotech, USA). Values were normalized to the corresponding expression of HPRT, a housekeeping gene used as internal control.

Quantitative PCR (RT-PCR^q): Total RNA was isolated from both experimental and control groups using GeneElute mammalian total RNA Miniprep Kit (Cat # RTN-70, Sigma, USA) and checked for purity and yield with the help of Nanodrop ND-1000 Spectrophotometer V3.3 (Nanodrop Technologies Inc., Wilmington, DE, USA). The quality of RNA was also assessed by running onto 2% denaturing agarose gel. Total RNA (1µg) was reverse-transcribed into cDNA by SuperScript III first strand cDNA synthesis Kit (Cat #18080-051, Invitrogen Life Science, USA) using random hexamer primers. Quantitative Real Time PCR was performed using ABI 7900HT Sequence Detection System (Applied Biosystems, USA). Real time reactions were carried out with Power SYBR Green PCR Kit (M/s Applied Biosystems, USA) as per the protocol provided by manufacturer. For PCR amplification, an initial denaturation was performed at 95°C for 15 min followed by 40 cycles of denaturation (95°C for 15sec) and annealing and extension (60°C for 60sec). PCRs were carried out in triplicates for each sample. Dissociation reaction was also carried out for each primer to confirm that only one product is formed by one primer. Further, samples were electrophoresed in 2.0% agarose gel to ensure that nonspecific amplification did not occur. Specificity of the each primer was assessed by melting curve analysis and NTCs for respective primer. Hypoxanthine-phosphoribosyltransferase (HPRT) was used as internal control to normalize the data and OGD induced alterations in mRNA expression are expressed in relative quantity (RQ) compared with respective unexposed control groups. Primer sequences used in the study are available in Table 3.1.

Table 3.1. Oligonucleotide Primer Sequences.

S.no	Target genes	Primer sequences	References
1.	Bim	Forward: 5'-AGTCTCAGGAGGAACCTGAAGATCT-3' Reverse: 5'-TCCGATCCTCCGCAGCT-3'	Näpänkangas et al., 2003
2.	Cavbeta3	Forward: 5'-GGCACTTCTGTCCCATCAGC-3' Reverse: 5'-CACCCGCACTGCTCCTGT-3'	Chameau et al., 2006
3.	P21 Accession no. U24174	Forward: 5'-GAGGCCTCTTCCCCATCTTCT-3' Reverse: 5'AATTAAGACACACTGAATGAAGGCTAAG3'	Self Designed (Primer Express 3.0 (App.Biosys., USA).
4.	MMP11 Accession no. U46034	Forward: 5'-GATGGAGGCCAGCTAGTCAG-3' Reverse: 5'-ATGGTACATGACCACGCAGA-3'	Mitsios et al., 2007
5.	HIF-1α	Forward: 5'-GTTTACTAAAGGACAAGTCACC-3' Reverse: 5'-TTCTGTTTGTTGAAGGGAG-3'	Acosta-Iborra et al., 2009
6.	GAPDH	Forward: 5'-AAGCTGGTCATCAATGGGAAAC-3' Reverse: 5'-GAAGACGCCAGTAGACTCCACG-3'	Leutenegger et al., 1999
7.	HPRT	Forward: 5'-CCAGTCAACGGGCGATATAA-3' Reverse: 5'-CTTGACCAAGGAAAGCAAGG-3'	Wen et al., 2006
8.	STAT 3 Accession no. NM012747	Forward: 5'-TCCTGGTATCCCCACTGGTC-3' Reverse: 5'-TTCCGAATGCCTCCTCCTT-3'	Self Designed (Primer Express 3.0 (App.Biosys., USA).
9.	Hsp27 Acession no. S 67755	Forward: 5'-ATCACTGGCAAGCACGAAGA-3' Reverse: 5'-GGGTGAAGCACCGAGAGAT-3'	-do-
10.	PTGER2 Accession no. NM031088	Forward: 5'-ACGAAAGCCCAGCCATCAG-3' Reverse: 5'-CAGCGCGATGAGGTTTCC-3'	-do-
11.	GSTP1 Accession no. N 012577	Forward: 5'-GTGCCCGCCCCAAGA T-3' Reverse: 5'-GATGGGACGGTTCAAATGGT-3'	-do-
12.	Bax	Forward: 5'GGGTGGTTGCCCTTTTCTACT-3' Reverse: 5' CCCGGAGGAAGTCCAGTGTC-3'	Singh et al., 2009
13.	Bax	Forward: 5'-TCCAGGATCGAGCAGA-3' Reverse: 5'-AAGTAGAAGAGGGCAACC-3'	Zhang et al., 2006
14.	Bcl_2	Forward: 5'-CTGTACGGCCCCAGCATGCG-3' Reverse: 5'-GCTTTGTTTCATGGTACATC-3'	Greenlund et al., 1995
15.	c-fos	Forward: 5'-GTAGAGCAGCTATCTCCTGA-3' Reverse: 5'-TCCACATCTGGCACAGAGC-3'	Pan et al., 2003
16.	c-jun	Forward: 5'-AACGACCTTCTACGACGATG-3' Reverse: 5'-GCAGCGTATTCTGGCTATGC-3'	Itoh et al., 2000
17.	GAP-43	Forward: 5'-GAGGGAGATGGCTCTGCTAC-3' Reverse: 5'-CACATCGGCTTGTTTAGGC-3'	Self Designed (Primer Express 3.0 (App.Biosys., USA).

1-12: Primer sequences used in Real-Time quantitative PCR using SYBR Green I Dye.
13-17: Primer sequences used in Reverse Transcriptase semi quantitative PCR.

Translational studies

OGD and reoxygenation induced alteration, and curcumin and *trans* resveratrol mediated restoration in the protein expression of marker genes known to be associated with ischemic cerebral stroke were carried out using immuno-cytochemical

localization and immuno-western blotting with the help of specific monoclonal antibodies.

Immunocytochemical quantification of marker proteins: Immunocytochemical localization of gene proteins viz., c-fos, c-jun, Bax, Bcl-2 and GAP-43 was done by the protocol of Kapoor *et al.* (2007) with desired modifications. In brief, cells (1 x 10^4 cells / well) were allowed to adhere on Poly-L-Lysin pre-coated eight well chamber slide flasks. The cells were then subjected to receive OGD and reoxygenation insult and treatment of drugs as described earlier. Following experimental schedule, cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. The cells were then washed with PBS thrice (10 minute for each wash) and incubated for 15 minutes in PBS containing 0.02% Triton-X100 and 0.1% BSA to block the non-specific binding. Cells were again washed with PBS thrice and incubated with primary monoclonal antibodies (1:200) at room temperature for 2 h in orbital shaker. Following incubation, cells were washed with PBS thrice to remove the unbound antibodies. Then HRP conjugated secondary antibody (goat anti rabbit, Calbiochem, USA) was added to each well and kept on a rocker shaker for 1 h at room temperature (antibodies were diluted in blocking buffer). Finally, cells were washed with PBS thrice and incubated with DAB (diamino bezidiene tetrahydrochloride, Sigma, USA) for 5-15 minutes to develop the colour. Cells were observed for protein localization under upright fluorescence cum phase contrast microscope (Nicon 80i, Japan) and images were grabbed using CCD cool Nicon DS-Ri-112.1 MP camera hooked with microscope. Up or down regulations in marker protein were calculated by measuring the change percent area of protein expression with the help of Leica Qwin 500 Image Analysis Software (Leica, Germany).

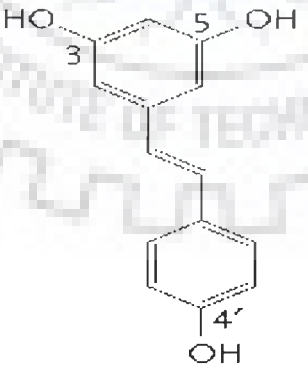
Western blot analysis: Western blot analysis for the expression of dopamine DA-D_2 receptor, c-fos, c-jun, bcl-2, bax and GAP-43 was done following standard protocol. In brief, cells from respective groups were pelleted and lysed using CelLytic™ M Cell Lysis Reagent (Catalog No# C2978, Sigma, USA) in the presence of 1X protein inhibitor cocktail (Catalog No# P8340, Sigma, USA). After protein estimation by BCA Protein Assay (Catalog No# G1002, Lamda Biotech, Inc., St. Louise, MO, USA), equal amount (50 µg/well) of proteins were loaded in 10% Tricine-SDS gel (Hermann Schagger *et al.*, 2006) and blotted on polyvinylidene fluoride (PVDF) membrane at 250 mA for 2 h by wet transfer system. After blocking for 2 h at 37°C, the membranes

were incubated overnight at 4°C with anti-protein primary antibodies specific for Bax, c-jun and c-fos (1:500, Santa Cruz, USA), Bcl_2, (1:1000, CST, USA) and GAP-43 (1:2000, Santa Cruz, USA) in blocking buffer (pH 7.5). The membrane was then incubated for 2 h at room temperature with secondary anti-primary immunoglobulin G (IgG) conjugated with horseradish peroxidase (Calbiochem, USA). Then, the blots were developed using either by luminol (Cat. No. 34080, Thermo Scientific, USA) or by TMB-H_2O_2 (Sigma, USA) and densitometry for protein specific bands was done in Gel Documentation System (Alpha Innotech, USA) with the help of AlphaEase™ FC StandAlone V. 4.0.0 software.

Anti-stroke potential of drugs

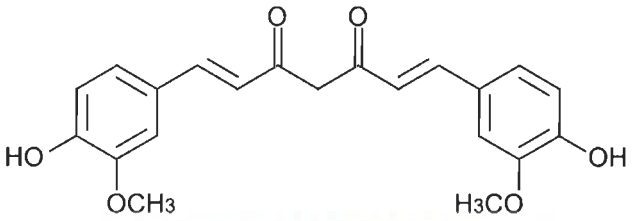
PC12-OGD *in vitro* model of ischemic stroke developed during the study was used to assess the anti-stroke potential of two drugs viz., *trans* resveratrol and curcumin, which are known to have neuroprotective activity. Both *trans* resveratrol and curcumin, used in the study were of highest purity available and procured commercially from Sigma-Aldrich, USA.

***Trans* resveratrol:** Red wine contain both *cis* and *trans* isomers of resveratrol with trans form in significantly higher concentration. The chemical structure of resveratrol has a striking similarity to the synthetic estrogen diethylstilbestrol. Two phenol rings are linked by a styrene double bond to generate 3,4′,5-trihydroxystilbene. Resveratrol exists as *cis* and *trans* isomeric forms, with *trans* to *cis* isomerization facilitated by UV exposure. *Trans* resveratrol (MW=228) is commercially available and is relatively stable if protected from high pH and light.



Structure of resveratrol.

Curcumin: Curcumin is one among the most established polyphenolic compounds containing drug known for a wide therapeutic window by its strong anti-inflammatory, anti-oxidant, thrombolytic and anti-carcinogenic activities.



Structure of curcumin.

Treatment schedule: Prior to use in the experiments, biological safe doses of both *trans* resveratrol and curcumin were determined using standard endpoints of cytotoxicity assessment i.e. trypan blue dye exclusion assay, tetrazolium bromide salt MTT assay, neutral red uptake assay and lactate dehydrogenase assay. The concentrations of *trans* resveratrol and curcumin selected in the study were 5, 10 and 25 μM and 100, 200 and 400 μM respectively. Batches of cells showing 95% or more viability were used in the study. The cells were divided into five groups, i.e. (1) pre-treatment, (2) post-treatment, (3) whole-treatment (4) OGD control, and (5) basal control group as depicted in Fig. 3.2. In pre-treatment group, cells were treated with either drug for 24 h prior to OGD, followed by 6 h of OGD and 24 h of reoxygenation. In post-treatment group, cells were treated with drug during reoxygenation period (24 h) only. In the whole-treatment group, cells were treated with the drug for the entire period of experiment i.e. 24 h prior to OGD, during 6 h of OGD and 24 h of reoxygenation. OGD control group were not given any drug treatment whereas in normoxia basal control group, cells neither received OGD insult nor treated with any drug. On completion of reoxygenation period, cells from both experimental and control group were harvested by centrifugation at 1000 rpm for 6 min. As per the demand, the OGD insult and drug treatment to the cells were given in different working formats i.e., 96/48/24/12/6 well culture plates and culture flasks.

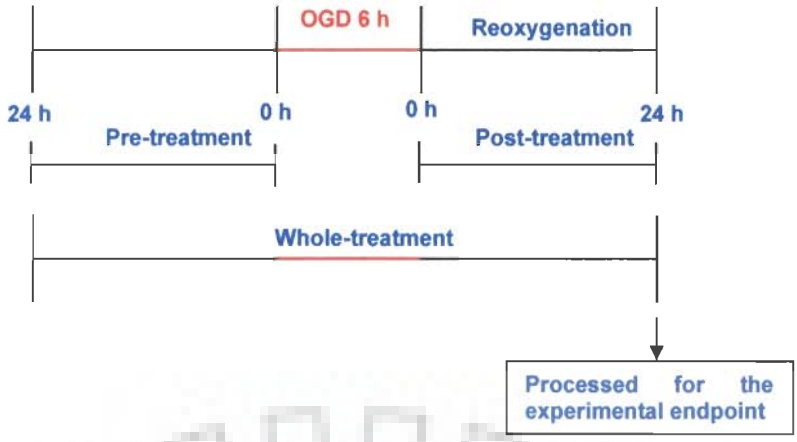


Fig. 3.2 Diagrammatic representation of treatment schedule (*in vitro* studies).

***In vivo* studies in rat middle cerebral artery occlusion (MCAo) model of ischemic cerebral stroke**

Animals: Albino male Wistar rats weighing 230-250 g were used as MCAo model of stroke. The animals were procured from the central animal house facility of Indian Institute of Toxicology Research, Lucknow and stock bred in the divisional (Development Toxicology Division) animal house. The rats were group housed in polyacrylic cages (38×23×10 cm) with not more than 4 animals per cage and maintained under standard laboratory conditions with natural dark and light cycle. They were allowed free access to standard dry rat diet and tap water *ad libitum*. However, 12 h before the behavioral testing, the rats were deprived of food as this is known to enhance their motivation to perform the test.

Ethical clearance for animal experimentation: All protocols involving the use of experimental animals were reviewed by Institutional Animal Ethical Committee of Indian Institute of Toxicology Research, Lucknow and animal experimentation was carried out only after receiving the ethical clearance.

Treatment schedule: Experimental animals were divided into 5 groups i.e. pre-, post- and whole-treatment groups, vehicle and sham control group. Oral feeding of the test drug (*trans* resveratrol) was done at a dose of 20 mg/kg daily for 7 days prior to MCAo (pre-treatment group), 7 days during reperfusion (post-treatment group) and for 14 days i.e., 7 days prior to till the end of reperfusion period (whole-treatment group) (Fig. 3.3a). Vehicle control (gum acacia) and sham control groups were also run under

identical conditions. Following exposure to *trans* resveratrol, animals were subjected to carryout the endpoints (listed in Fig. 3.3b) by day 1 and day 7 of reperfusion period.

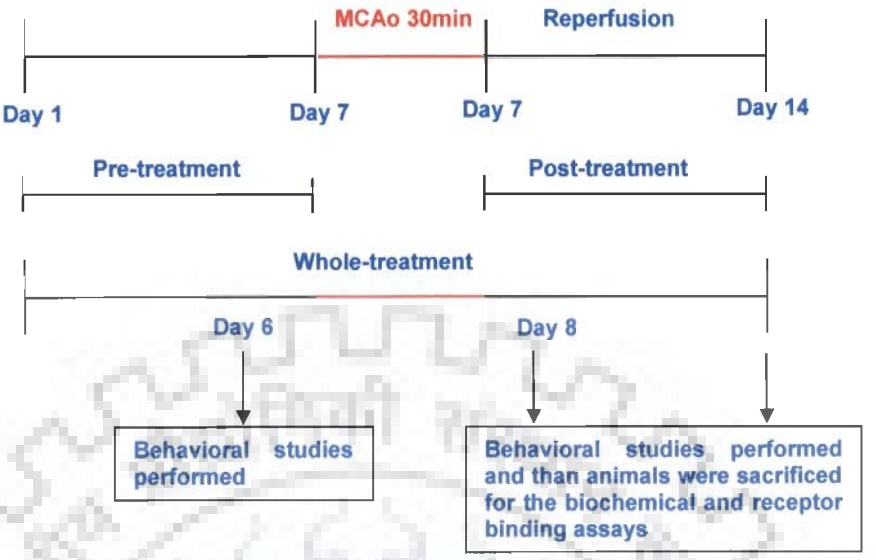


Fig. 3.3a Diagrammatic representation of treatment schedule (*in vivo* studies).

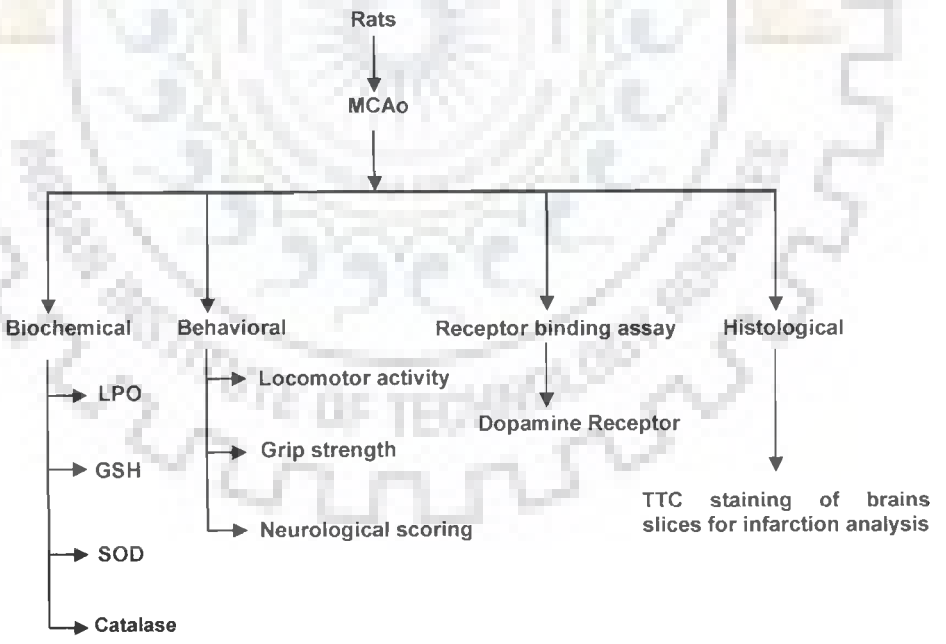


Fig. 3.3b Endpoints selected for *in vivo* studies.

Induction of focal cerebral ischemia in rats: Focal cerebral ischemia was induced in rats by occlusion of middle cerebral artery. Rats were anesthetized by administration of chloral hydrate (350 mg/kg, i.p.) and then mounted on the dissecting table. After shaving the neck region, a midline incision was made and the right common carotid artery was dissected until the bifurcation of the common carotid into the external carotid artery and internal carotid artery. External carotid artery was ligated at two sites and a cut was made between the two so that the artery could be pulled down towards the heart. Thereafter, a nick was made in the external carotid artery near the proximal ligature and a 3.0 monofilament nylon thread (Ethicon, Johnson & Johnson, USA) with its tip rounded by heating near a flame and coated with poly-lysine, was advanced from the external carotid artery into the lumen of the internal carotid artery until resistance was felt which ensures the occlusion of the origin of middle cerebral artery. The nylon filament was allowed to remain in the place for 30 min. The neck incision was closed by suturing the muscles using catgut and the skin by sterilized nylon thread. On the completion of ischemia of 30 min, the filament was retracted so as to allow reperfusion of the ischemic region (Gupta *et al.*, 2005). Fig. 3.4 shows the branches of the common carotid artery and the path followed by the intraluminal thread to block the middle cerebral artery. The body temperature of the animals was kept maintained with the help of heating lamps during the whole procedure.

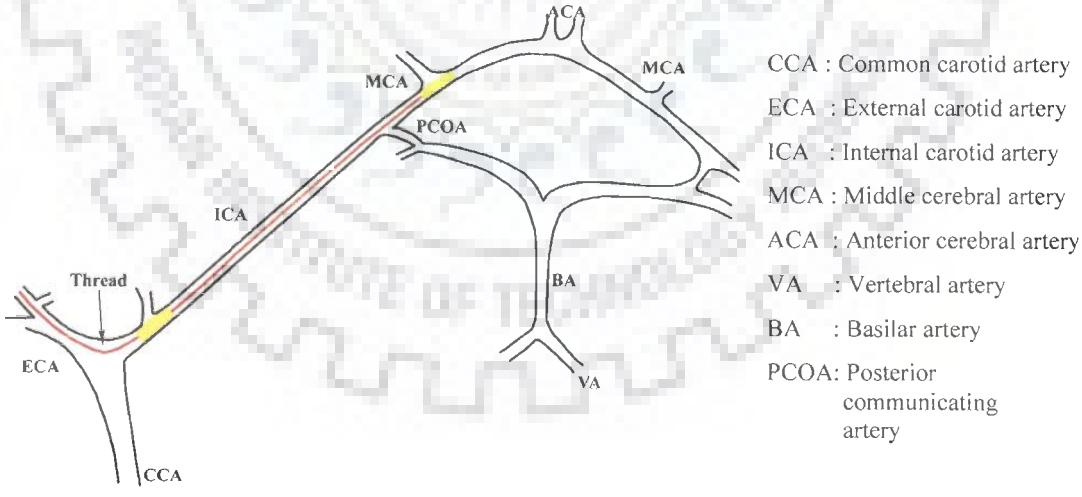


Fig. 3.4 Branches of the common carotid artery (CCA) and the path followed by the intraluminal thread to block the middle cerebral artery (MCA).

Postoperative care of animals: After carrying out the surgical procedure, neosporine powder was applied on the incision site. The rats were then housed in individual cages washed with antiseptic lotion and bedded with sterilized cotton. The animals were monitored till their recovery from anesthesia. After recovery the rats were fed with dextrose and shifted to clean cages padded with sterile husk.

Body weight response: Systemically healthy male Wistar albino rats weighted between 230 to 250 g were selected in the study. Animals (n = 7 in each group) were divided into six groups viz., normal control (without any surgery), sham-operated (surgery without MCAo), vehicle treated control (surgery and MCAo), pre treated (*trans* resveratrol treatment for seven days prior to MCAo), post treated (*trans* resveratrol treatment for seven days after MCAo) and whole treated (*trans* resveratrol treatment for seven days prior to seven days after the MCAo) group. Their weights were recorded.

Monitoring of motor activity

To study the protective potential of *trans* resveratrol, animals of different group viz., drug treated and vehicle control were subjected for motor activity at 24 h and 7 days after middle cerebral artery occlusion using battery of tests i.e. neurological scoring, grip strength and closed field activity tests. Sham group was also kept in the study as internal control.

Neurological scoring: To ascertain the neuronal health, animals were observed for 15 minutes in observation chamber and only healthy animals were selected for experiments. After MCAo, both control and treated groups were observed (at 24 h and 7 days of MCAo) for neurological deficit that was graded on 6-point scale proposed by Tatlisumak *et al.* (1998) as follows:

0 = no deficits

1 = failure to extend left forepaw fully

2 = circling to the left

3 = paresis to the left

4 = no spontaneous walking

5 = death

Grip strength: Forelimb grip strength was measured in control, sham operated and MCA occluded rats using software guided grip strength meter (TSE, Germany)

following the procedure as described by Dunnett *et al.* (1998). The apparatus was consisted of a force sensor module with trapezoidal grip of dimensions 60 x 30 mm, 2 mm diameter, elevated 60 mm from a flat surface. Rats were gently held by the nape of neck and the base of the tail. The forelimbs of the rats were placed on the tension bar and the rat was pulled back gently until it released the bar. The readings (in pounds) were recorded on the computer.

Locomotor activity: Spontaneous locomotor activity was monitored in a computerized Optovarimax system (Columbus Instruments, Columbus, OH, USA) following the method of Ali *et al.* (1990) with required modifications. The Optovarimax is a horizontal two-dimensional activity meter and consists of two arrays of 15 infrared beams placed perpendicular to each other. The beams are spaced approximately 25.4 mm apart and each beam is very narrow (3 mm in diameter). Rats were individually placed in the test apparatus, acclimatized for a period of 2 min and their locomotor activities were recorded for 5 min. Interruption in the photo beams positioned in parallel inside the chamber resulted in an activity count in terms of distance traveled, which was processed by the microcomputer and recorded for data analysis.

Oxidative stress analysis

Following the treatment schedule, one set of animals from each group were sacrificed (at 24 h and 7 days after MCAo) by cervical dislocation and their brains were dissected out immediately, cleaned by rinsing with chilled saline and processed for lysate preparation following the methodology described by Gupta *et al.* (2005). Processed brain samples were analyzed for protein contents following the protocol of Lowery *et al.,* (1951) with desired modifications. Samples were kept in -80°C and used for the analysis within 48 h. The endpoints studied to estimate the oxidative stress were malondialdehyde (MDA), reduced glutathione (GSH), catalase and superoxide dismutase (SOD). Restorative/protective potential of *trans* resveratrol was assessed by comparing the data of treated groups with vehicle and sham controls.

Malondialdehyde (MDA) levels: Malondialdehyde, indicator of lipid peroxidation, was estimated as originally described by Okhawa *et al.,* (1979) and modified by Gupta *et al.,* (2005). Brain tissues were homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). The reagents, acetic acid 1.5 ml (20%, pH 3.5), 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulfate (8.1%) were added to 0.1

ml of processed tissue sample. The mixture was heated at 100°C for 60 min and then cooled in tap water. 5 ml of n-butanol: pyridine (15:1 v/v) and 1 ml of distilled water was added to it and vigorous mixing was done through vortaxing. After centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn and absorbance was measured at 532 nm using spectrophotometer (Cary 300 Bio, USA). Standard curve was plotted using external standard i.e., tetra methoxy propane (1-10 nmol). The concentration of MDA was determined by the linear standard curve.

Glutathione content (GSH): Reduced glutathione was estimated by the method described by Ellman (1959). Brain tissues were homogenized as in case of malondialdehyde assay. This homogenate was then centrifuged with 5% trichloroacetic acid to separate out the proteins. Homogenate (0.1 ml) was mixed with 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5'5-dithiobis-(2-nitrobenzoic acid) (DTNB) and 0.4 ml of double distilled water. The mixture was vortexed and kept at room temperature for 15 min prior to being read at 412 nm. Parallel set with standard glutathione (1-10 μg) were also run under identical conditions and used to plot the standard curve against the concentration of reduced glutathione. The concentration of GSH was determined by the linear standard graph.

Superoxide dismutase (SOD) activity: Superoxide dismutase activity was estimated as described by Kakkar *et al.* (1984). Homogenized brain tissue (0.1 ml) was mixed thoroughly with sodium pyrophosphate buffer 1.2 ml (0.052 M, pH 8.3), 0.1 ml phenazine methosulphate (186 μM), 0.3 ml nitro blue tetrazolium (300 μM) and 0.2 ml NADH (780 μM) and incubated for 90 min at 30°C. Following incubation, it was mixed with 4 ml n-butanol and 1 ml acetic acid. The reaction mixture was then centrifuged at 4000 rpm for 10 min, the organic layer was withdrawn and absorbance was measured at 560 nm using spectrophotometer (Cary 300 Bio, USA). A similar reaction was also run parallel with standard superoxide dismutase (2-10 U) and standard curve was plotted which served as internal control. The concentration of SOD was determined by the standard graph.

Catalase activity: Catalase activity was estimated following the method of Sinha, (1972) with required modifications. 1 ml (100 times diluted) brain homogenate was used as starting material, which was mixed with 0.1 ml of phosphate buffer (pH 7.4) and 0.4 ml of distilled water. Reaction was started by addition of 0.5 ml H_2O_2 (0.2 M)

solution and incubated for 1 min at room temperature. The reaction was stopped by addition of 2 ml of potassium dichromate-acetic acid reagent (5% potassium dichromate solution mixed in glacial acetic acid in 1:3) and kept for 15 min in boiling water bath. The mixture was then cooled and read at 570 nm against control. Internal control set was run under identical conditions without the addition of H_2O_2.

Dopamine (DA-D_2) receptor analysis: Following MCA occlusion at 24 h and 7 days, one set of animals were sacrificed by cervical dislocation and brains were dissected out immediately. After rinsing of the brains with pre-chilled 0.32 M sucrose solution, they were processed for homogenization. Brains were homogenized in a ratio of 1:19 in pre-chilled 0.32 M sucrose and centrifuged at 50000 × g for 10 min. The pellets were re-suspended and homogenized in 5 mM Tris-HCl buffer (pH 7.4) in the same volume and centrifuged again at same speed for 10 min at 4°C. The pellets were finally suspended in 40 mm Tris-HCl buffer (pH 7.4) and stored at -80°C till used. The radioligand binding studies were carried out following the protocol as described for PC12 cells under *in vitro* section.

Infarction studies in brain slices with TTC staining

Infarction analysis was performed following the protocol standardized in the laboratory by Shukla and Khanna, (2008). Five coronal sections (2 mm thick) of the whole brain were taken from the region beginning 1 mm from the frontal pole and ending just rostral to the cortico-cerebellar junction and stained with 2% 2,3,5-triphenyl tetrazolium chloride (TTC) solution in saline for 10 min at 37°C. After fixing in 10% formaldehyde overnight, slices were scanned using flat bed scanner. The area of infarction was measured in coronal brain sections using Leica Qwin 500 image analysis software (Leica, Germany). Infarct areas of all sections were cumulated to get total infarct area which was multiplied by thickness of brain sections to get the volume of infarction.

Statistical analysis

Unless otherwise stated, all the results were calculated as means ± SD. Statistical difference between the two mean values were determined by student's t-test for unpaired observations. Analysis of variance (ANOVA) was used to find the statistical difference if more than two groups were compared, followed by a Tukey test. All the straight lines (of standard curves) were drawn by linear regression and test

for linearity done. For neurological deficits, the values are represented as median ± 95% confidence intervals. $p < 0.05$ represents level of significance.

Results

4. RESULTS

NGF induced differentiation

PC12 cells were found to be differentiated into neuronal like morphology and physiology following continuous supplementation of NGF (50 ng/ml) for 8 days. Most of the cells displayed typical neural cell morphology with developed dendrites by day 8. No further morphological differentiation could be recorded with further exposure of NGF i.e. for 16 and 21 days. A gradual shrinkage in dendritic structures could be recorded in 8 days differentiated cells kept in culture medium without NGF for further period i.e. 16 and 21 days whereas, cells kept in NGF supplemented medium remained differentiated till 21 days (Photograph 4.1).

Expression of neuronal specific marker genes

Along with morphological differentiation, PC12 cells were also found to have physiological expression of marker gene proteins viz., nestin, β-tubulin, neurofilament L (NF-L), neurofilament M (NF-M), neuritin 5 and dopamine DA-D_2 receptor since day zero of differentiation. The magnitude of expression increased with the extended differentiation period i.e. up to 8 days. However, the expression of NF-L and NF-M could be detected from day 2 onwards of differentiation. After day 8 of differentiation, a non-significant increase in the magnitude of expression of these neuronal markers was observed. These differentiated neuronal cells were found to be healthy with the expression of all the neuronal markers (Photograph 4.2).

Optimization of oxygen glucose deprivation (OGD) conditions

Optimum time points for oxygen-glucose deprivation (OGD) and reoxygenation have been identified to suggest the suitability of PC12 cells as rapid and sensitive *in vitro* model of cerebral stroke. Further, the precise role of glucose as one of the limiting factors was ascertained. PC12 cells were subjected to OGD of 1-8 h followed by reoxygenation for 6 to 96 h in culture medium having glucose 0-10 mg/ml. Loss of cell viability was assessed using trypan blue dye exclusion and MTT assays.

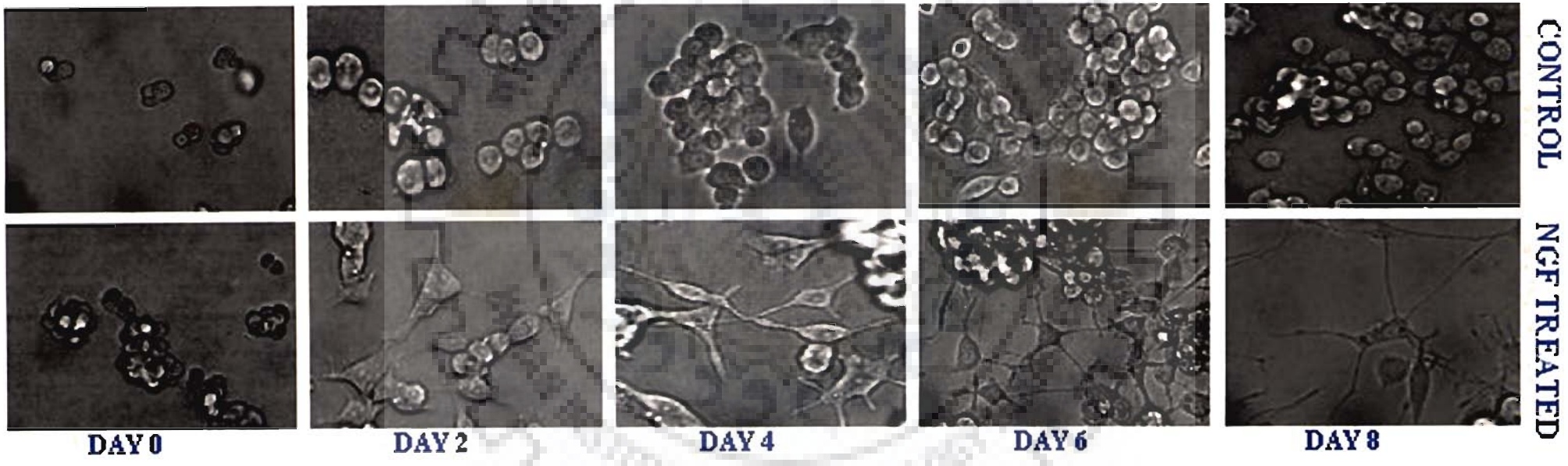
Effect of OGD on cell viability: Loss in viable cell count was assessed immediately after the OGD at each interval (1 h interval up to 8 h) using trypan blue dye exclusion test. The results are summarized in Fig. 4.1. At the start of experiment, under normoxia condition (0 h), the percent cell viability could be recorded as 97.0±0.6 of counted

cells. Statistically significant ($p<0.05$) reduction in percent viable cell count started at 2 h of OGD (80.7±2.0). This gradual decrease in percent cell viability continued in all further OGD periods (3, 4, 5, 6, 7 and 8 h) i.e., 65.7±3.5, 59.7±4.6, 54.3±3.2, 44.7±2.9, 20.3±4.3, 5.7±2.0 of counted cells respectively. All these reductions were comparable to the values of normoxia (0 h) and were of highly significant range ($p<0.001$). However, decrease in cell viability was not significant following OGD of 4 h compared with 5 h and OGD of 5 h compared with 6 h, whereas this difference was statistically highly significant following OGD of 6 h when compared to OGD of 4 h ($p<0.01$). As per the recommendations of international regulatory agencies, loss of cell viability of more than 50% is considered as cytotoxic response, thus the OGD period for 6 h onwards falls under this category. Therefore, an OGD period of 6 h was selected for further experimentation to find out the optimum glucose concentrations in the culture medium and period of reoxygenation.

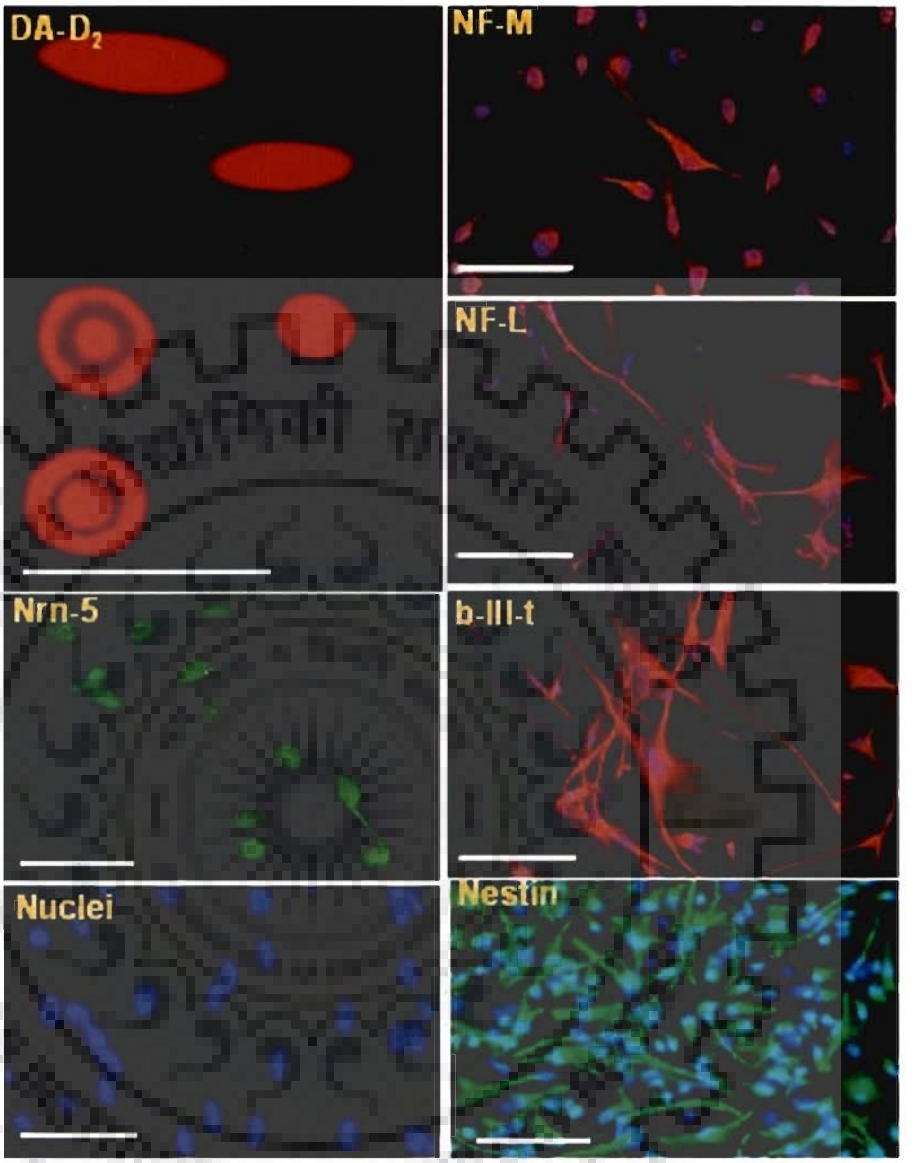
Influence of glucose concentrations during reoxygenation: MTT assay was conducted to evaluate the influence of glucose concentration used in the culture medium during reoxygenation period. The effect of various concentrations of glucose (0-10 mg/ml) was evaluated for total reoxygenation period up to 96 h at the intervals of 6, 12, 18, 24, 36, 48, 72 and 96 h following an OGD of 6 h. The highlights of the results are summarized in Fig. 4.2a-c. It is well evidenced from the data that starvation started even in first 6 h post OGD in the cells re-oxygenated in glucose free medium and a time-dependent gradual and statistically high significant ($p<0.001$) decrease in cell viability was recorded till the end of reoxygenation period i.e. 96 h (Fig. 4.2a).

During reoxygenation, a polyphasic response for percent cell viability in PC12 cells was observed. With an initial increase in concentration of glucose i.e. up to 4 mg/ml, a gradual increase in the cell viability was observed after which a stationary phase was observed between 4-6 mg/ml glucose concentrations (Fig. 4.2b). This was followed by a sequential decrease in cell viability with the increase in glucose concentrations up to the maximum concentration used in the study i.e. 10 mg/ml (Fig. 4.2c).

A maximum and almost similar level of influence of glucose could be recorded between 4-6 mg/ml at all the time intervals selected in the study with maximum effect at 24 h (Fig. 4.3). Thus, the glucose concentrations between 4-6 mg/ml could be



Photograph 4.1: Nerve growth factor (NGF) induced differentiating PC12 cells



Photograph 4.2: Protein expression of neural specific marker genes in PC12 cells

DA-D2: Dopamine -D_2 receptor
NF-M: Neurofilament medium
NF-L: Neurofilament lower
b-III tubulin
Nrn-5: Neuritin 5
Scale bar: 50mm
Original magnification: 400X

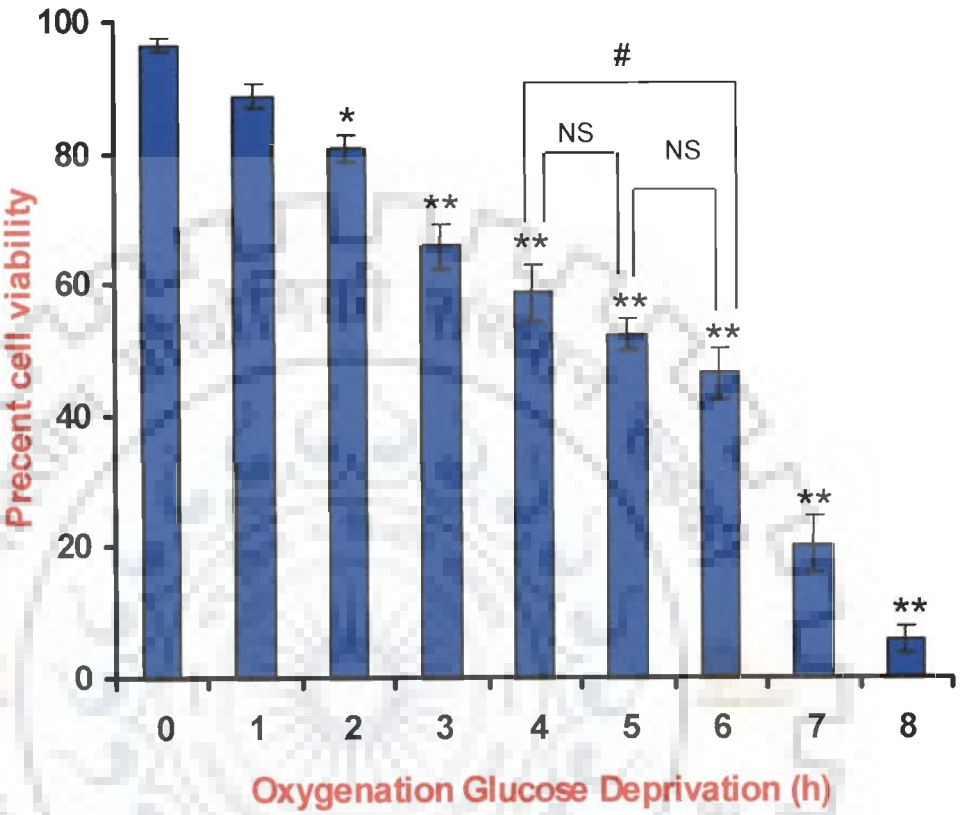


Fig. 4.1: **Percent cell viability in cultured PC12 cells subjected to OGD insult for variable periods**

*p<0.05, **p<0.001 in comparison to the cell viability at 0 h.
#p<0.05 when cell viability following OGD of 4 or 6 h were compared.
NS: not significant, when cell viability following OGD of 4 or 5 h and 5 or 6 h were comparedz

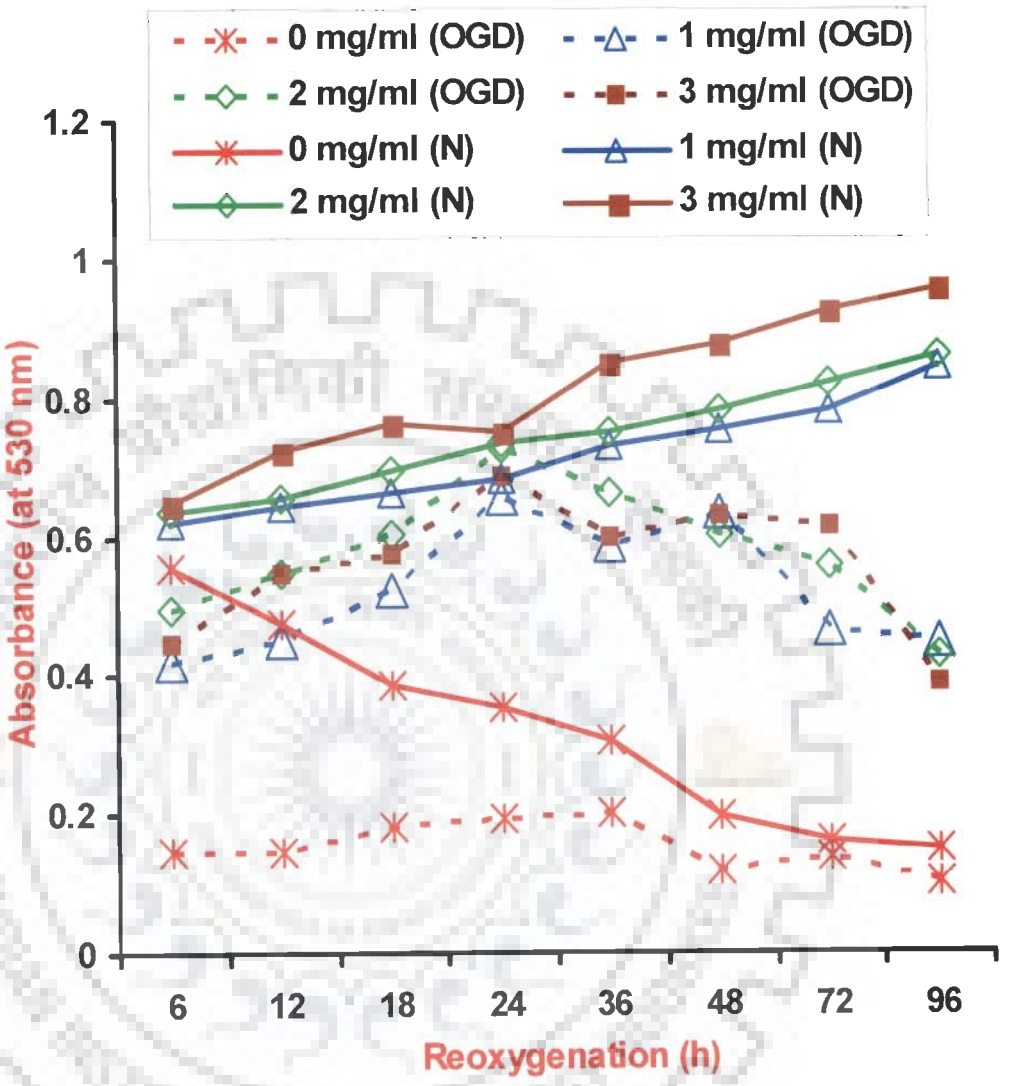


Fig. 4.2a: **Response of glucose concentration (0-3mg/ml) on growth pattern at various time points during reoxygenation in cultured PC12 cells subjected to OGD of 6 h**

N: Normoxia; OGD: Oxygen glucose deprivation

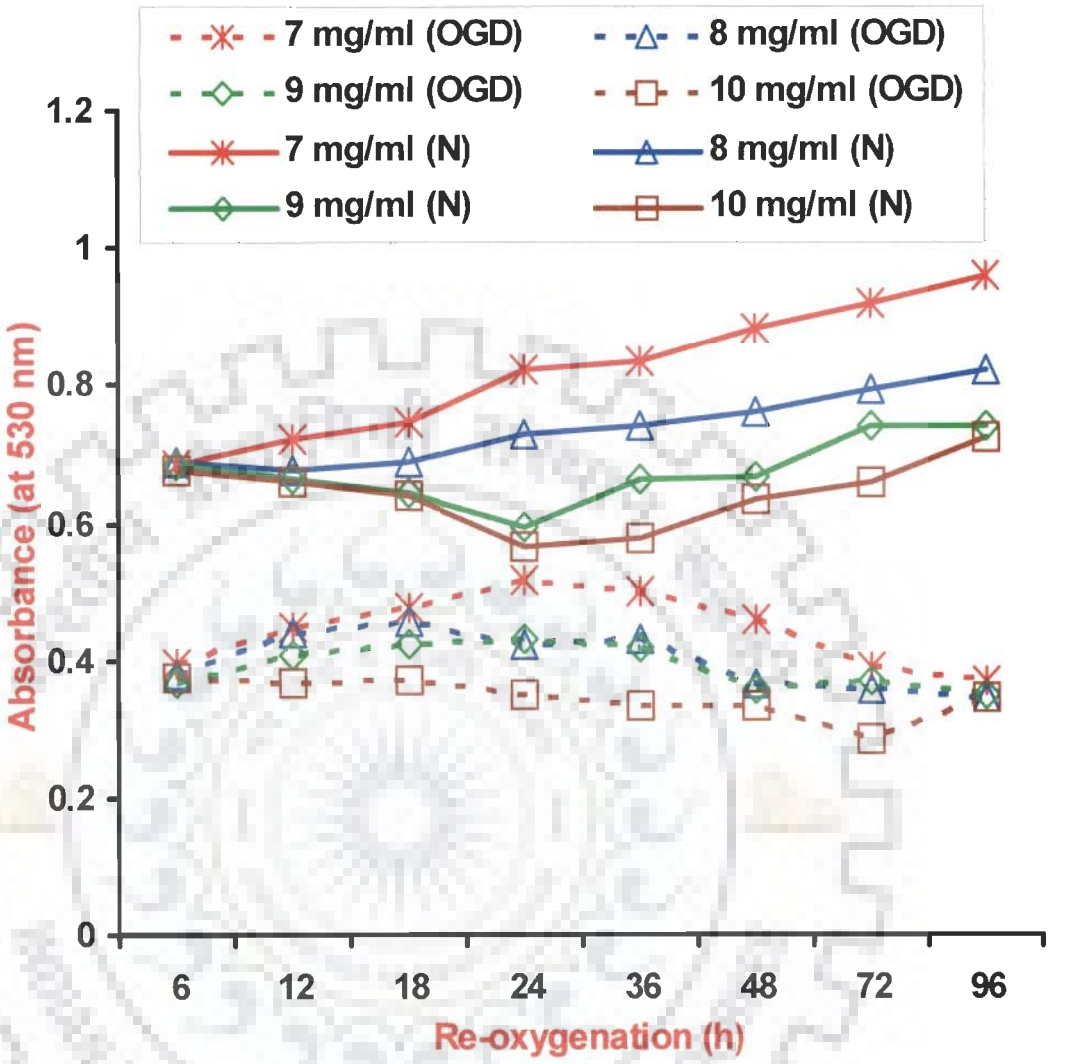


Fig. 4.2c: **Response of glucose concentration (7-10mg/ml) on growth pattern at various time points during reoxygenation in cultured PC12 cells subjected to OGD of 6 h**

N: Normoxia; OGD: Oxygen glucose deprivation

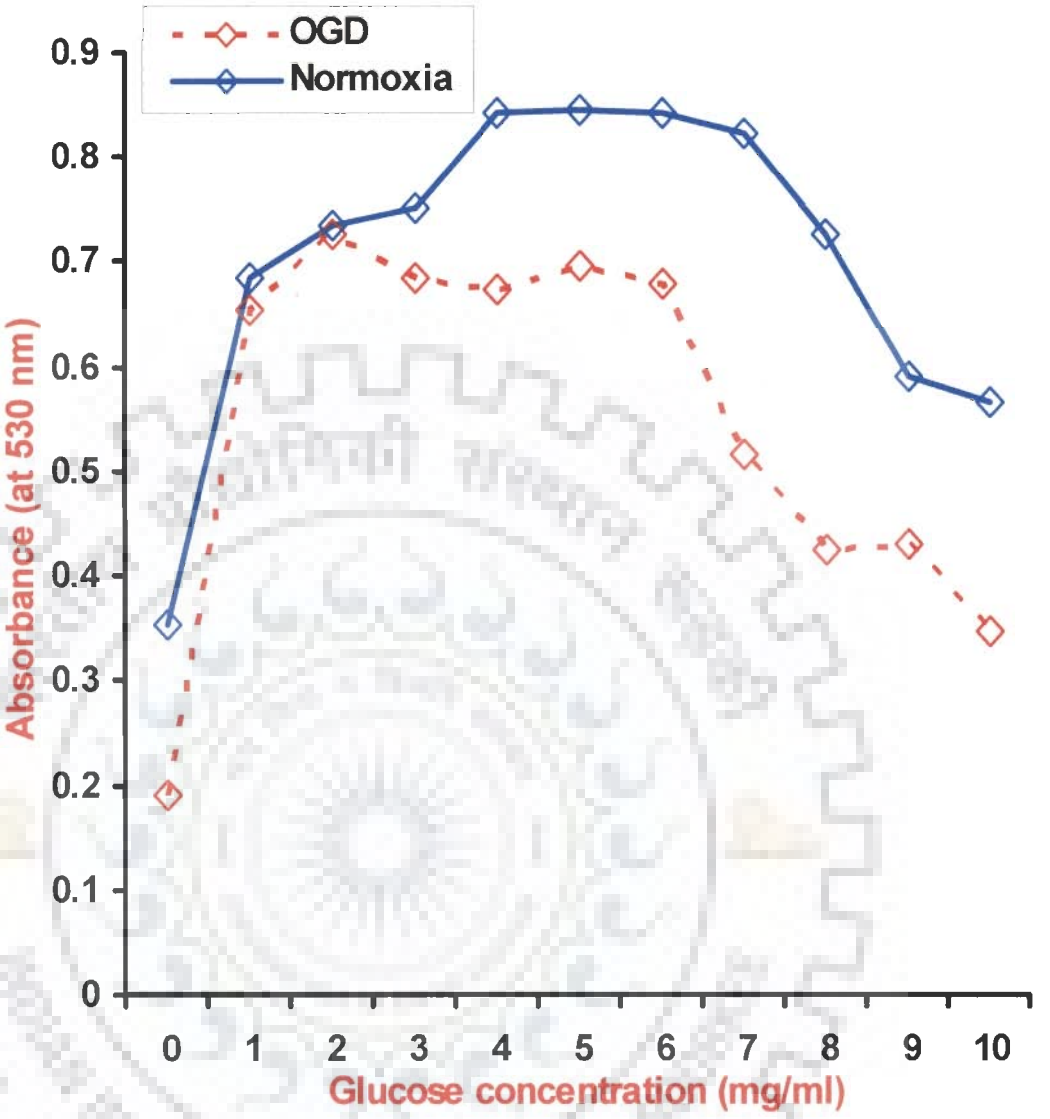


Fig. 4.3: **Growth pattern post 24 h of reoxygenation in PC12 cells subjected to 6 h of OGD insult vs normoxic cells under the influence of various concentrations of glucose**

suggested as the optimum concentrations in the medium to which cells should be exposed during reoxygenation period.

Time period and glucose concentrations during reoxygenation period: Summary of two dimensional studies to identify the combination of best reoxygenation period and most effective concentration of glucose in culture medium is presented in Fig. 4.2a-c and Fig. 4.3. A gradual growth response could be recorded at each interval during reoxygenation period i.e. 6, 12, 18, 24, 36, 48, 72 and 96 h. However, this response could not attain statistically significant levels till 18 h of reoxygenation when compared with the cell viability at 6 h during reoxygenation. Statistically significant changes were prominent from 24 h of reoxygenation and continued till the end i.e. 96 h when compared with the cell viability at 6 h of reoxygenation. In the inter-group comparison, reoxygenation of 24, 36 and 48 h were found to be statistically insignificant. Similarly, difference in cell viability at 72 and 96 h of reoxygenation was not significant whereas the cell viability at 24, 36 and 48 h was statistically high significant when compared with both 72 and 96 h. In general, at all the time intervals during reoxygenation period, maximum cell viability was observed in culture medium containing glucose concentration between 4-6 mg/ml. The difference in cell viability under the influence of 4, 5 and 6 mg/ml was statistically insignificant at all the time intervals of reoxygenation. Reoxygenation period of 24 h was found to be first statistically significant time point for all the glucose concentrations. It was selected as reoxygenation period for all further studies. Also, there was no significant difference in cell viability after 24 h of reoxygenation in all three groups of glucose concentration (Fig. 4.2b).

Glucose concentration during reoxygenation was found to be one of the key factors involved in the growth and proliferation in PC12 cells. The OGD of 6 h followed by a reoxygenation period of 24 h with 4-6 mg/ml glucose concentration could be recorded as optimum conditions under the experimental setup and used as basic standards for all the further experiments.

Development of PC12 cells-OGD *in vitro* model of cerebral stroke and evaluation of anti-stroke potential of *trans* resveratrol and curcumin

To evaluate the suitability of PC12 cells as rapid and sensitive *in vitro* model of cerebral stroke, experiments were conducted to study the endpoints involved in cascade of events following OGD. Further, anti-stroke potential of known

neuroprotectives viz., *trans* resveratrol and curcumin was evaluated under identical experimental setup.

Mitochondrial activity: Results of the percent change in mitochondrial activity in different groups are summarized in Fig. 4.4a-b. Following the OGD insult (6 h) and reoxygenation (24 h), mitochondrial activity decreased to 75.3±3.1% when compared to the normoxia control cells. In general, pre-treatment group for both the drugs were found to have better protective response.

In case of *trans* resveratrol, a statistically significant restoration could be recorded in pre-treatment group at lower concentrations used i.e., 5 and 10 μM (108.9±5.1, 111.6±4.6%) followed by 25μM (128.1±3.3) when compared with the values observed in cells kept under normoxia. All the concentrations used were found to cause cytostatic response in post-treatment group as mitochondrial activity increased to 158.46±6.1, 164.9±5.1 and 176.1±5.4% of normoxia control following the treatment of 5, 10 and 25 μM of *trans* resveratrol respectively. The increase in the mitochondrial activity was statistically insignificant in whole-treatment group except for 5μM (133.2±2.7% of normoxia control) concentration of *trans* resveratrol. Thus, pretreatment of *trans* resveratrol may have some protective effect on PC12 cells subjected to prior OGD insult (Fig. 4.4a).

Like *trans* resveratrol, pre-treatment with curcumin was also found to be effective since it resulted in elevation of mitochondrial activity to 114.5±5.1, 100.2±4.6, 92.6±3.3% of normoxia control following treatment with 100, 200 and 400 μM curcumin respectively. In post-testament group, all concentrations were found to be cytostatic in a concentration dependent manner. However, in reverse order of concentration used i.e. 400 μM could be recorded as minimum cytostatic (170.6±6.2%), 200 μM (155.5±5.4%) moderate and 100μM (142.3±5.1%) maximum cytostatic. Surprisingly, a concentration-dependent deteriorating response [100μM (72.6±2.7%), 200 μM (56.6±1.9%), and 400 μM (49.3±1.9%)] was recorded in the whole-treatment group, when compared with OGD group (Fig. 4.4b). In totality, curcumin was found to have better activity than *trans* resveratrol in PC12 cells-OGD under the experimental conditions.

Intracellular calcium levels: Results for the intracellular calcium concentrations following OGD insult and reoxygenation and restorative potential of *trans* resveratrol

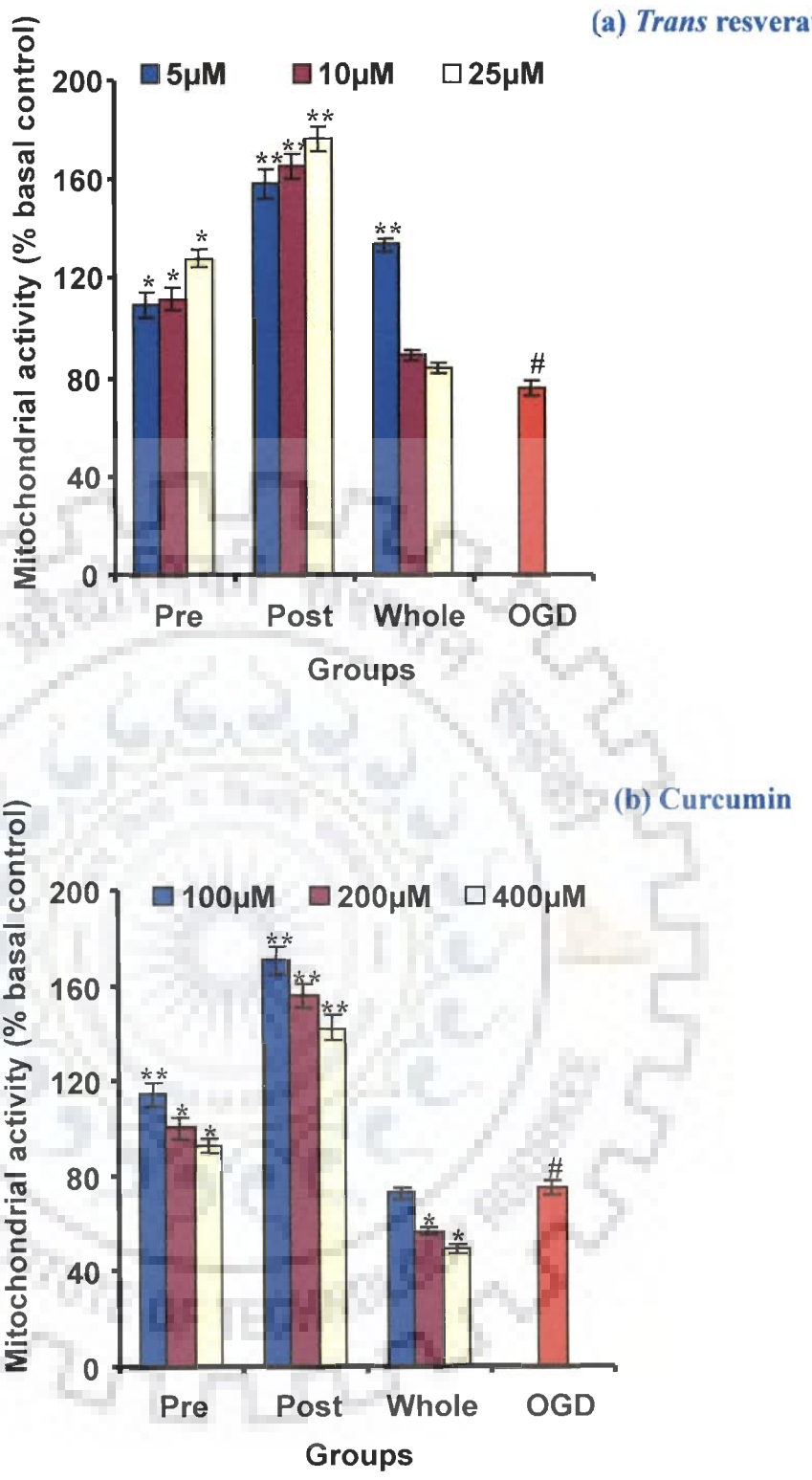


Fig. 4.4a-b: Mitochondrial activity in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
*p<0.05, **p<0.01 when compared with OGD

and curcumin are presented in Fig. 4.5a-b. Increase in intracellular calcium levels is one among the events known due to depolarization of the membrane. Under the experimental setup, PC12 cells of OGD group have shown statistically high significant increase in the intracellular calcium i.e., 343.6±35.2% of normoxia control group. All concentrations of *trans* resveratrol used in pre-treatment group exhibited statistically significant reduction in the calcium levels when compared with the OGD group. However, the intracellular elevated calcium levels were still significantly higher than normoxia control group. At highest concentration (25 μM) in post and whole-treatment groups the values reached near to the basal levels (121.7±17.1 and 143.4±15.3% of normoxia control respectively). Other concentrations of *trans* resveratrol used in these groups did not pose any significant effects (Fig. 4.5a).

Treatment with curcumin, in general, was observed to be effective in reducing intracellular calcium. As in case of *trans* resveratrol, the highest concentration (400 μM) of curcumin was found to reduce the level up to 121.7±14.5 and 121.9±13.5% of normoxia control in post and whole-treatment groups. Pre-treatment with curcumin at lower concentration 100 μM was found to be more effective (130.4±18.2% of normoxia control) then the higher concentrations (200 and 400 μM) used i.e. 191.3±19.6 and 195.6±20.1% respectively (Fig. 4.5b).

Membrane potential: The results for alteration in membrane potentials following OGD insult and reoxygenation and the restorative potential of *trans* resveratrol and curcumin are presented in Fig. 4.6a-b. It is apparent from the data that values for OGD (104.9±11.2%) and all the treatment groups were parallel to the normoxia control for both the drugs i.e. *trans* resveratrol and curcumin (Fig. 4.6a-b).

Lactate dehydrogenase (LDH) release: The results for the release of lactate dehydrogenase following OGD insult and reoxygenation and restorative potential of *trans* resveratrol and curcumin are presented in Fig. 4.7a-b. A significant increase in LDH release was observed in PC12 cells subjected to OGD i.e., 142.4±2.8% in comparison to normoxia control group.

Significant and more or less similar pattern of reduction in the LDH release could be recorded following both pre- and post-treatment of *trans* resveratrol in a concentration dependent manner. In the pre-treatment group, LDH values were 123.9±5.1, 116.1±4.8 and 111.9±3.6% in comparison to OGD group i.e. 142.4±2.8%

of normoxia control following the treatment of 5, 10 and 25 μM respectively. The values for post-treatment were 134.7±4.9, 127.3±4.7 and 116.7±5.7% following 5, 10 and 25 μM, respectively. The whole-treatment of *trans* resveratrol changes the behavior of cells in opposite direction and further increase the release of LDH greater then OGD group i.e. 147.4±5.2, 162.1±6.6 and 171.9±6.9% following 5, 10 and 25 μM respectively (Fig. 4.7a).

In case of LDH, curcumin was found to be comparatively more effective than *trans* resveratrol, however, the trend of reduction was similar and falls statistically highly significant for both pre and post-treatment groups. As in case of *trans* resveratrol, in the whole-treatment group with curcumin, LDH release was further increased with even greater magnitude i.e. 168.9±5.7, 187.6±6.3 and 196.5±7.9% following 100, 200 and 400 μM concentrations respectively when compared with OGD group i.e. 142.4±2.8% of normoxia control (Fig. 4.7b).

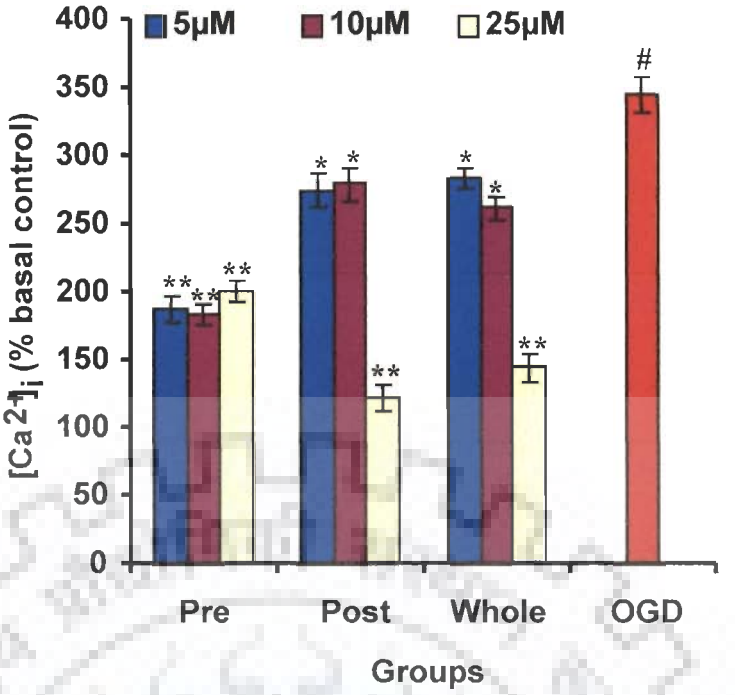
Prostaglandin E_2 (PGE_2): Inflammation has been reported to be one of the causative factors in the pathophysiology of cerebral ischemia and PGE_2 is one of the important mediators of inflammation. The results for the alterations in the levels of PGE_2 following the treatment schedule are presented in Fig. 4.8a-b. PGE_2 could be considered as one of the good endpoints using *in vitro* systems as values reached up to 161.7±14.8% of normoxia in OGD group. Both the drugs used in the study were found to reduce the levels of PGE_2 to or lower than the normoxia control group in all the treatment schedules. The magnitude of reduction in curcumin was found to be greater in comparison to *trans* resveratrol in pre-treatment group (Fig. 4.8a-b).

Oxidative stress

The results obtained for the alterations in the oxidative stress markers following OGD insult and reoxygenation and protective potential of two known antioxidants drugs i.e. *trans* resveratrol and curcumin are presented in Fig. 4.9-4.14a-b.

Lipid peroxidation (LPO): A statistically significant increase in the lipid peroxidation could be observed in OGD group i.e. 172.6±5.3% when compared with normoxia control group. In case of *trans* resveratrol, highest concentration i.e. 25 μM restored the values up to 130.3±4.5% of normoxia control in whole-treatment group only. The other concentrations and treatment schedules of *trans* resveratrol were found not so significantly effective (Fig. 4.9a).

(a) *Trans* resveratrol



(b) Curcumin

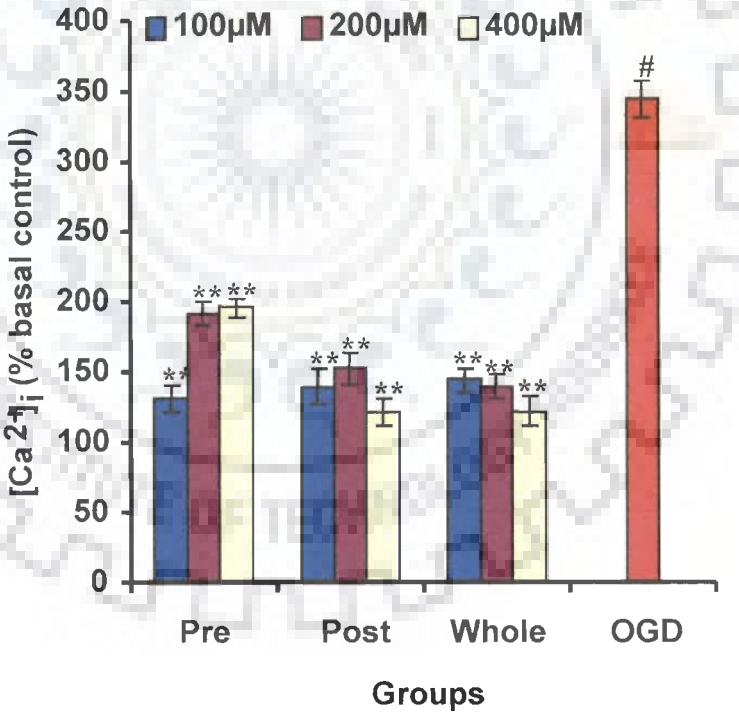


Fig. 4.5a-b: Intracellular calcium levels in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
*p<0.05, **p<0.01 when compared with OGD

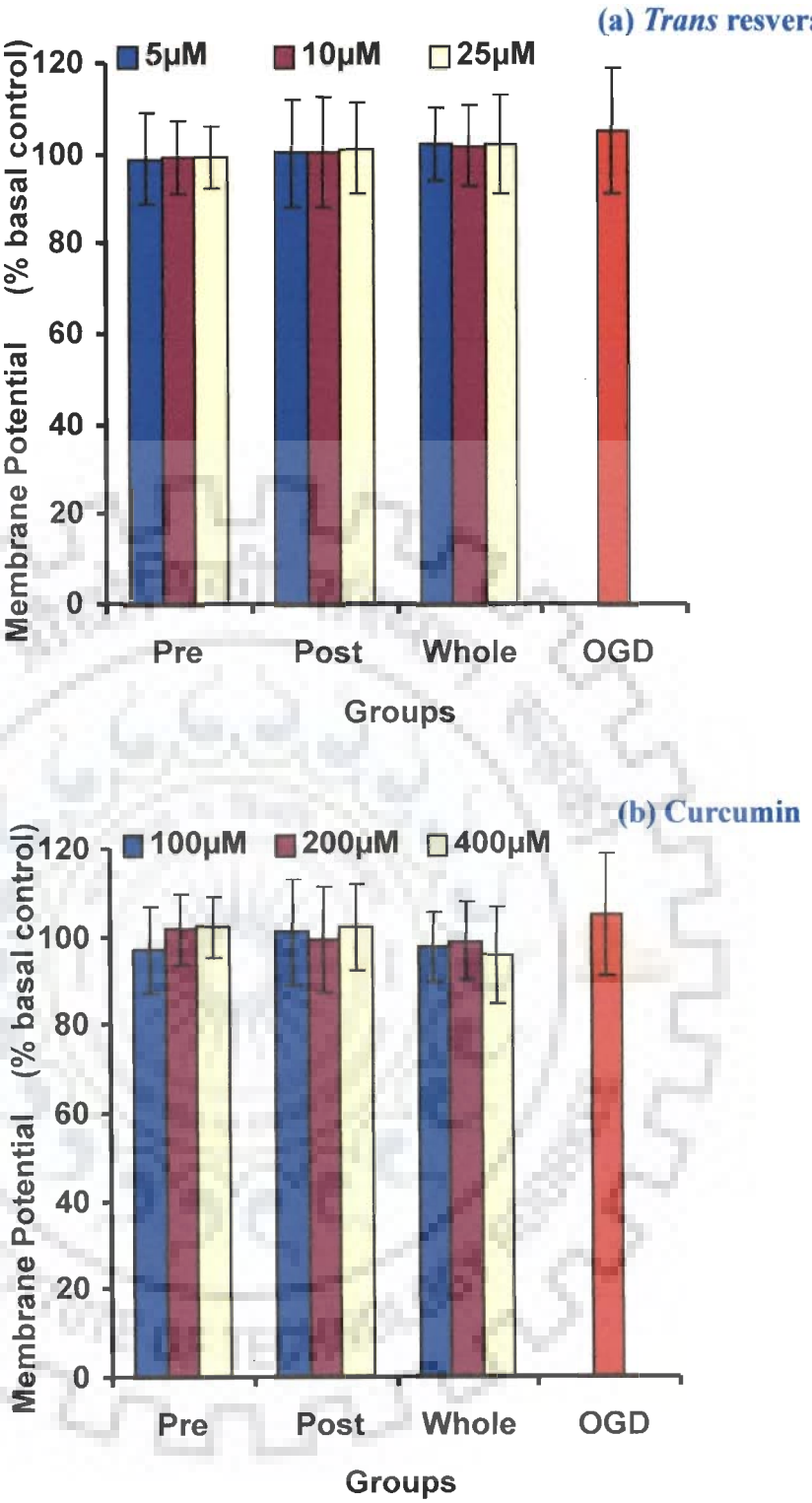


Fig. 4.6a-b: **Mitochondrial membrane potential in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment**

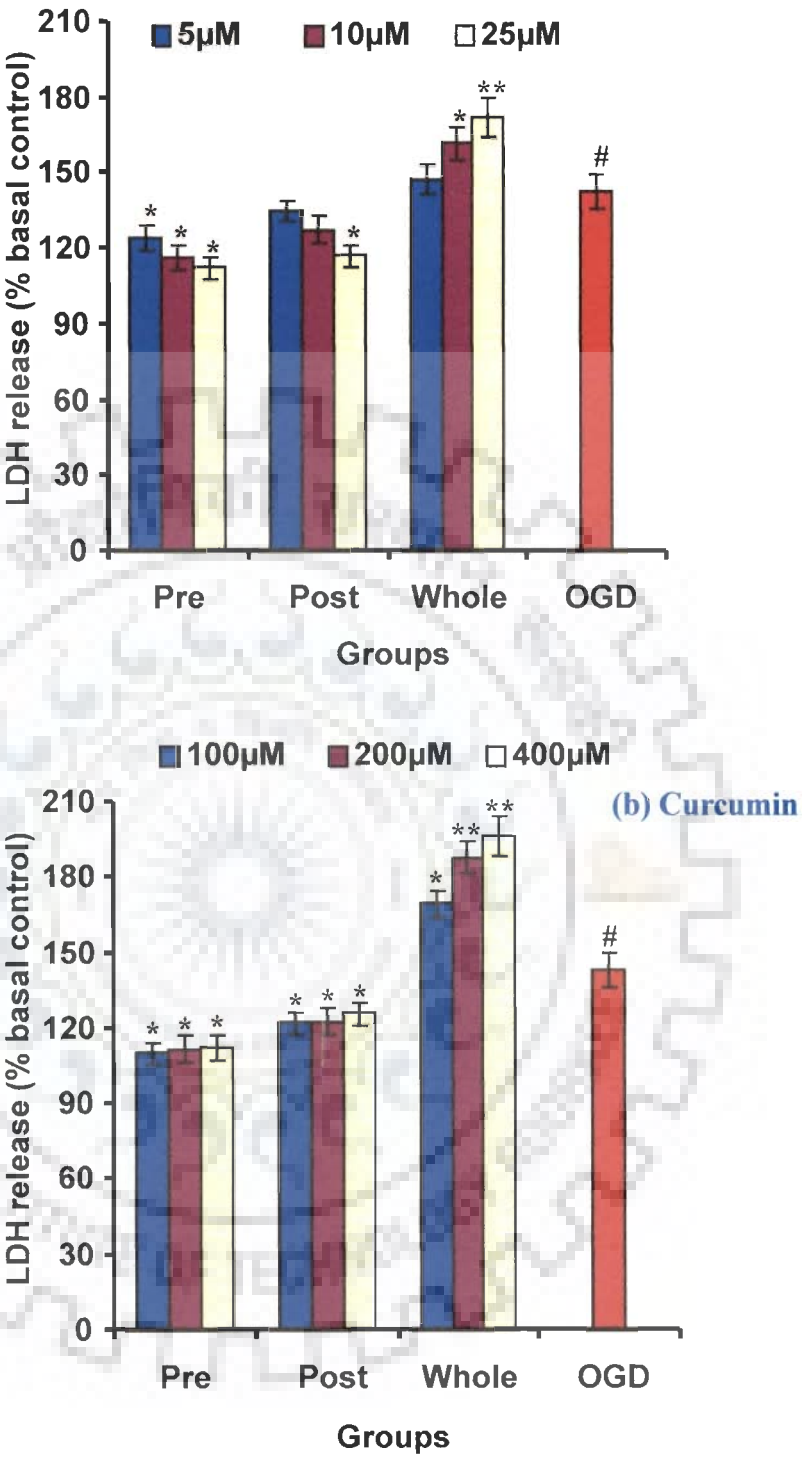


Fig. 4.7a-b: **Lactate dehydrogenase activity in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment**

#p<0.05 when OGD compared with basal control
*p<0.05, **p<0.01 when compared with OGD

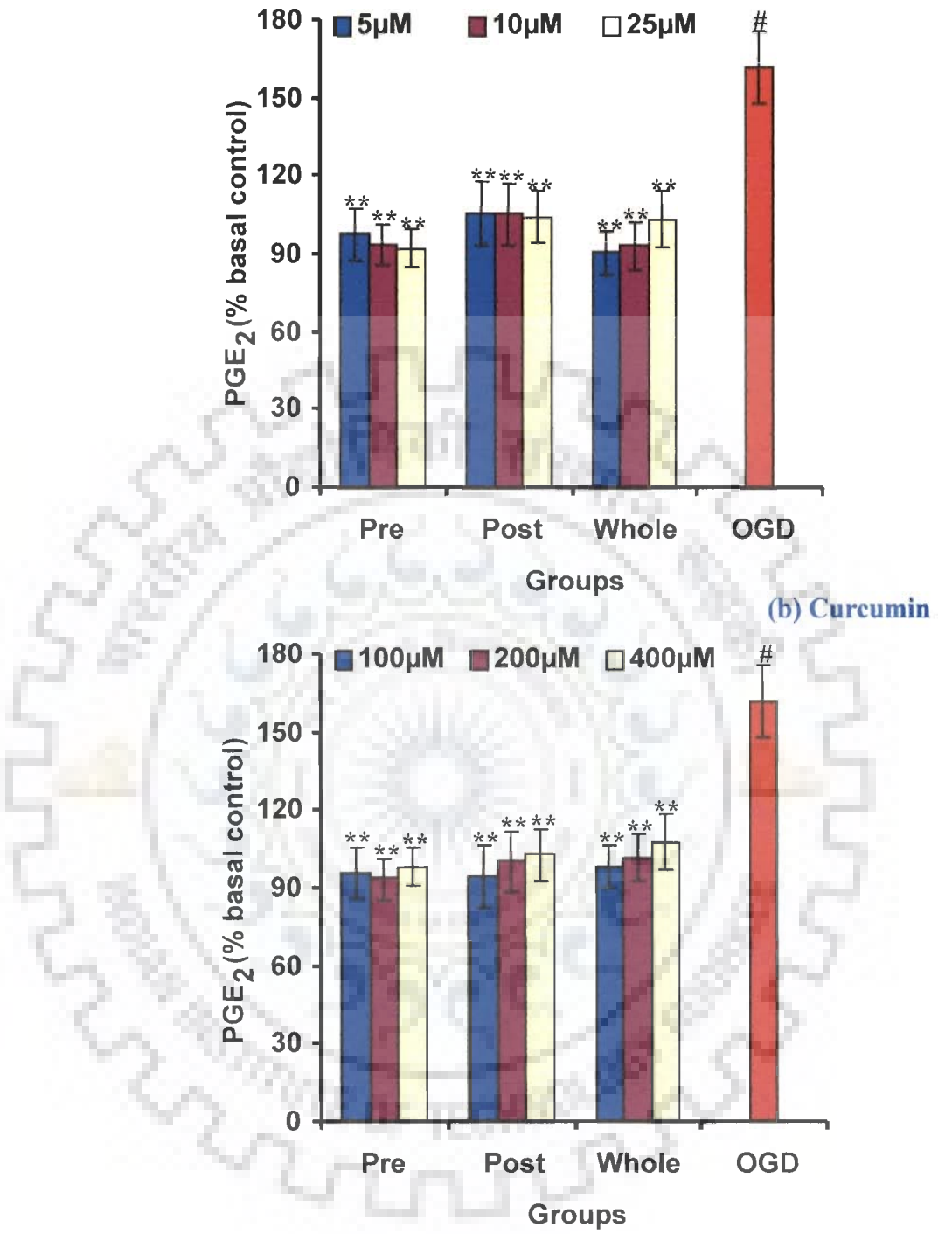


Fig. 4.8a-b: Prostaglandin E_2 release in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
**p<0.01 when compared with OGD

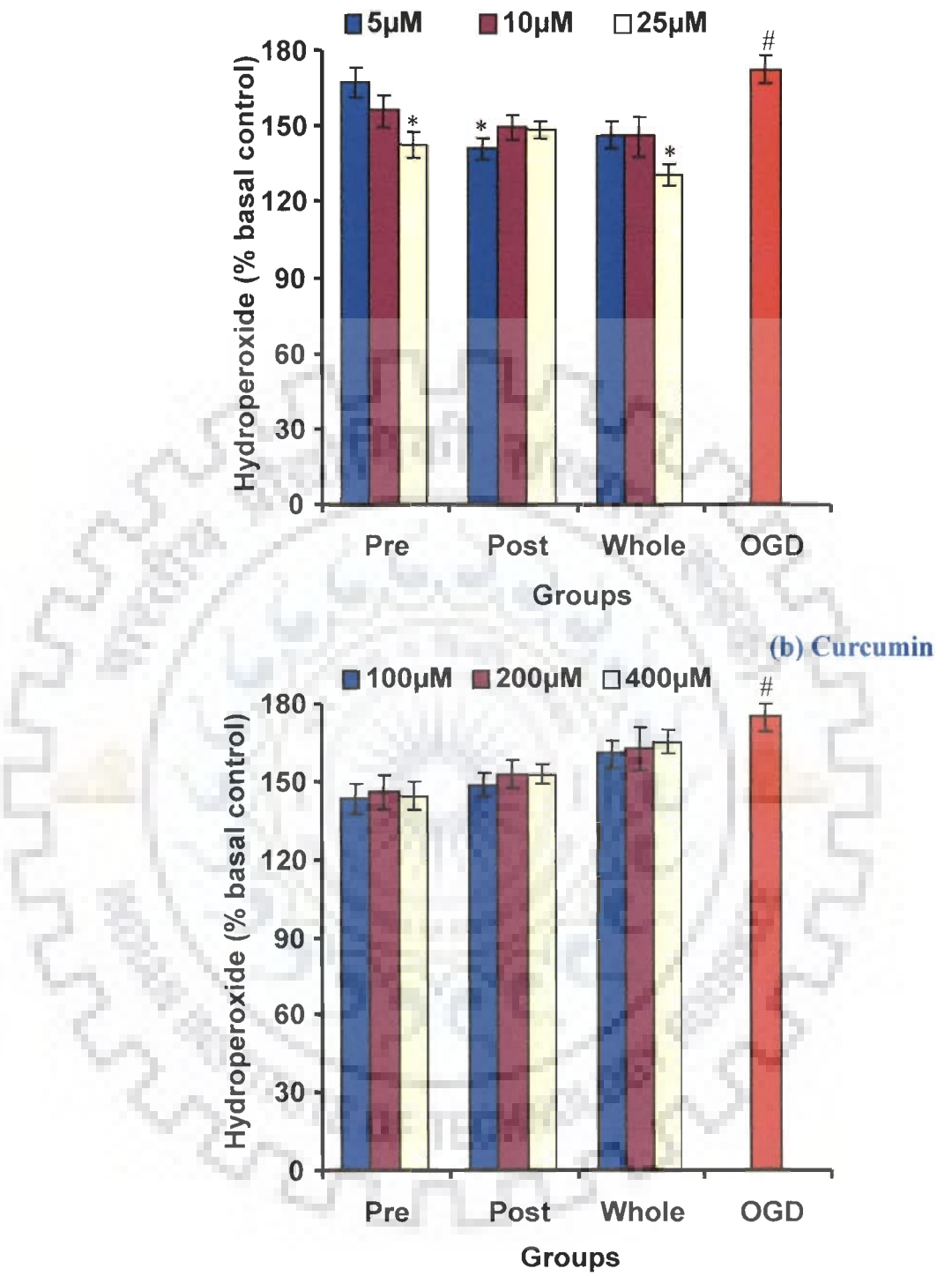


Fig. 4.9a-b: Lipid peroxidation in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
*p<0.05 when compared with OGD

None of the treatment schedule at any concentration of curcumin was found to have restorative potential for lipid peroxidation in OGD received PC12 cells post 24 h reoxygenation period (Fig. 4.9b).

Catalase activity: A significant decrease in the overall activity of catalase was observed that brought down to 61.2±3.5% of normoxia control in PC12 cells kept in OGD group. In general, no recovery in catalase activity could be recorded following the entire treatment schedule with any concentration of *trans* resveratrol except 25 μM (68.8±1.9%) in whole-treatment group (Fig. 4.10a).

In case of curcumin, pre-treatment schedule has shown a concentration dependent significant restoration in the catalase activity i.e. 88.4±2.9, 90.6±4.0 and 97.3±4.4% of normoxia control at 100, 200 and 400 μM respectively. A concentration dependent increase in the activity was also observed in post-treatment group, however it was not significant when compared with normoxia control except for highest concentration i.e. 400 μM (80.8±4.6%). Surprisingly, a concentration dependent deterioration in the catalase activity was observed in whole-treatment group that was maximum with 400 μM (52.4±1.9%) when compared with normoxia control (Fig. 4.10b).

Superoxide dismutase (SOD) activity: A significant decrease in the SOD activity was observed in the cells following 6 h OGD and 24 h reoxygenation (84.4±3.8%) when compared to normoxia control. Pre and post-treatment with *trans* resveratrol was found to be protective at all the concentration used, whereas in whole-treatment group, only 25 μM was able to increase the activity to some extent (97.6±5.8% of normoxia control) (Fig. 4.11a). Curcumin treatment in totality was found to be ineffective at all concentrations (Fig. 4.11b).

Glutathione (GSH) content: Glutathione, an endogenous antioxidant, is depleted significantly following OGD insult and reoxygenation (83.3±8.4% of normoxia control). Interestingly, 25 μM concentration of *trans* resveratrol could bring the level at par to the normoxia level in all the treatment schedules i.e. pre-treatment (112.2±11.3%), post-treatment (102.1±10.2%) and whole-treatment (118.3±11.5%) (Fig. 4.12a).

Moreover, a similar protective potential for curcumin was observed in pre and post-treatment groups. Following treatment, the glutathione levels recovered to reach

the absolute percentage to the normoxia control group. The recovery in whole-treatment group was insignificant except for 200 μM (93.3±9.1% of normoxia control) (Fig. 4.12b).

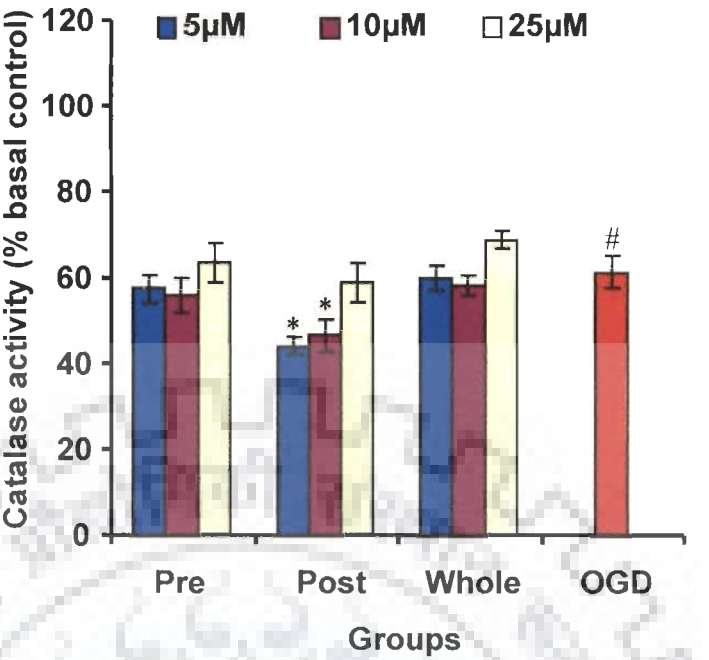
Reactive oxygen species (ROS) generation: More than two fold (230.4±13.6% of normoxia control) increase in the ROS production could be recorded in cells subjected to OGD (6 h) and reoxygenation (24 h). Although, the ROS generation was reduced significantly (192.2±12.5, 190.7±11.3 and 184.1±9.4% of normoxia control) following pre-treatment with *trans* resveratrol at concentrations 5, 10 and 25 μM respectively, still values were fairly higher then the normoxia control. Maximum efficacy (166.7±9.1% of normoxia control) of *trans* resveratrol could be observed only at highest concentration used i.e. 25 μM in whole-treatment group (Fig. 4.13a).

Pre-treatment with curcumin exhibited a drastic reduction in OGD-reoxygenation induced ROS generation in a concentration dependent manner and brought down the values parallel to the normoxia control i.e. 112.6±8.2, 105.6±7.5, and 101.5±5.4% following 100, 200 and 400 μM concentrations respectively. The post and whole-treatment groups of curcumin were also found to be effective in a concentration dependent manner but the values were much higher then the normoxia control. Treatment of curcumin appears to be better in controlling OGD and reoxygenation induced ROS generation then *trans* resveratrol (Fig. 4.13b).

Nitric oxide (NO) generation: A significant increase in the production of NO was observed following OGD insult that persisted till 24 h reoxygenation period (133.2±8.1% of normoxia control). The response of *trans* resveratrol treatment was statistically significant for all treatment schedules i.e. pre, post and whole-treatment, without much variation with the concentrations (Fig. 4.14a). In case of curcumin, only pre-treatment could bring down the values significantly i.e. 102.6±3.5, 103.9±3.4 and 105.6±4.2% of normoxia control following 100, 200 and 400 μM treatment respectively. Effects of post and whole-treatments of curcumin were found to be statistically insignificant (Fig. 4.14b).

Dopamine (DA-D_2) receptor analysis: Results of the alterations in DA-D_2 receptor levels following OGD-reoxygenation and treatment of *trans* resveratrol and curcumin are presented in Fig. 4.15a-b. DA-D_2 receptor levels were found to be decreased drastically in OGD group (51.6±2.4% of normoxia control). Pre-treatment with *trans*

(a) *Trans* resveratrol



(b) Curcumin

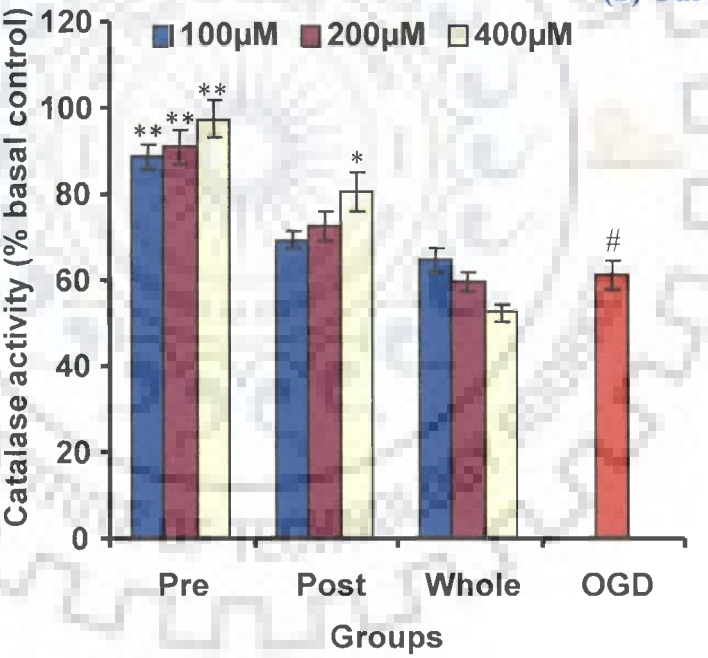
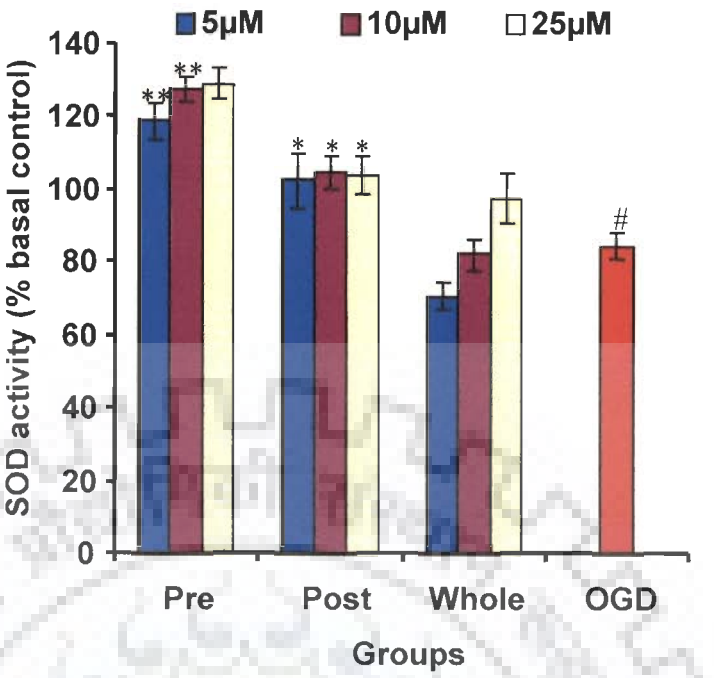


Fig. 4.10a-b: Catalase activity in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
*p<0.05, **p<0.01 when compared with OGD

(a) *Trans* resveratrol



(b) Curcumin

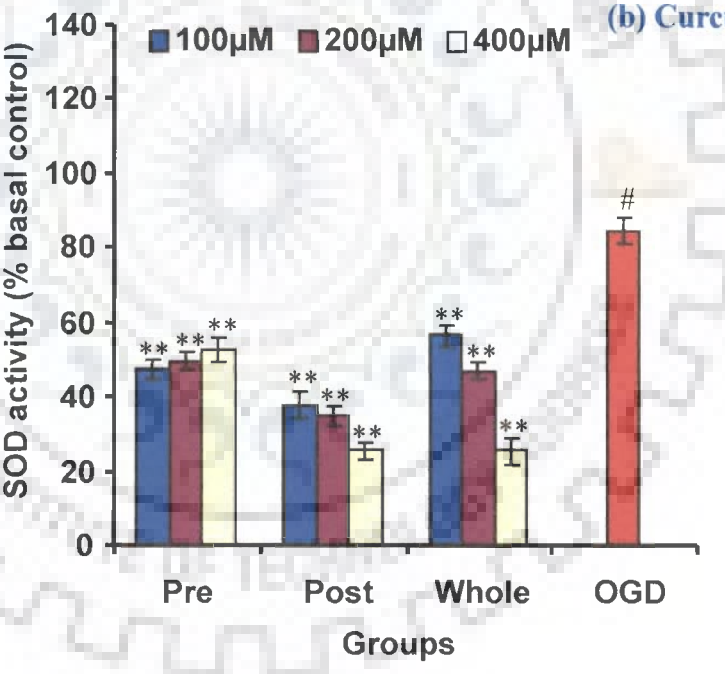
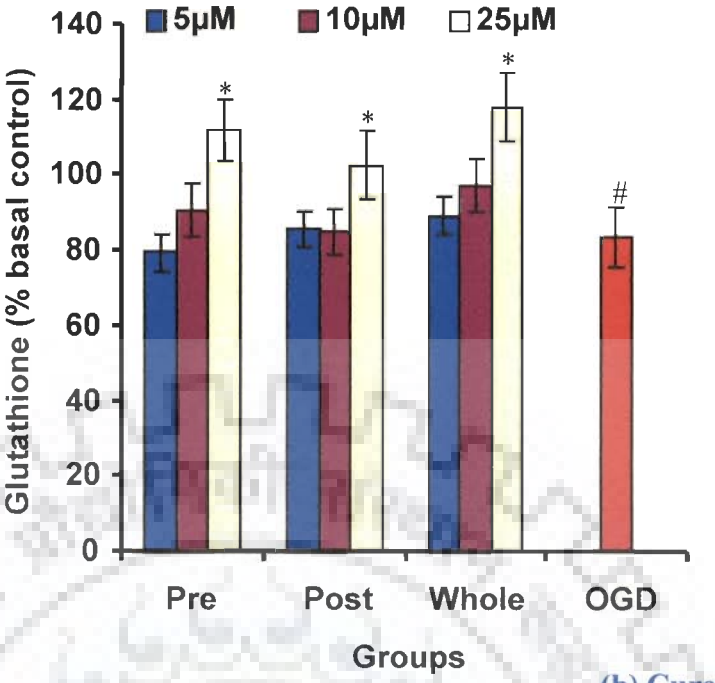


Fig. 4.11a-b: Superoxide dismutase activity in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
*p<0.05, **p<0.01 when compared with OGD

(a) *Trans* resveratrol



(b) Curcumin

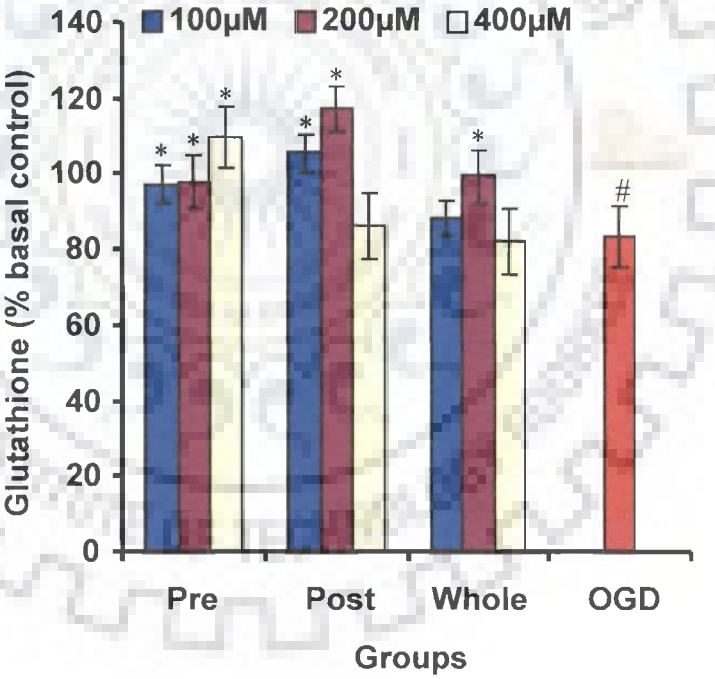


Fig. 4.12a-b: Glutathione levels in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
*p<0.05 when compared with OGD

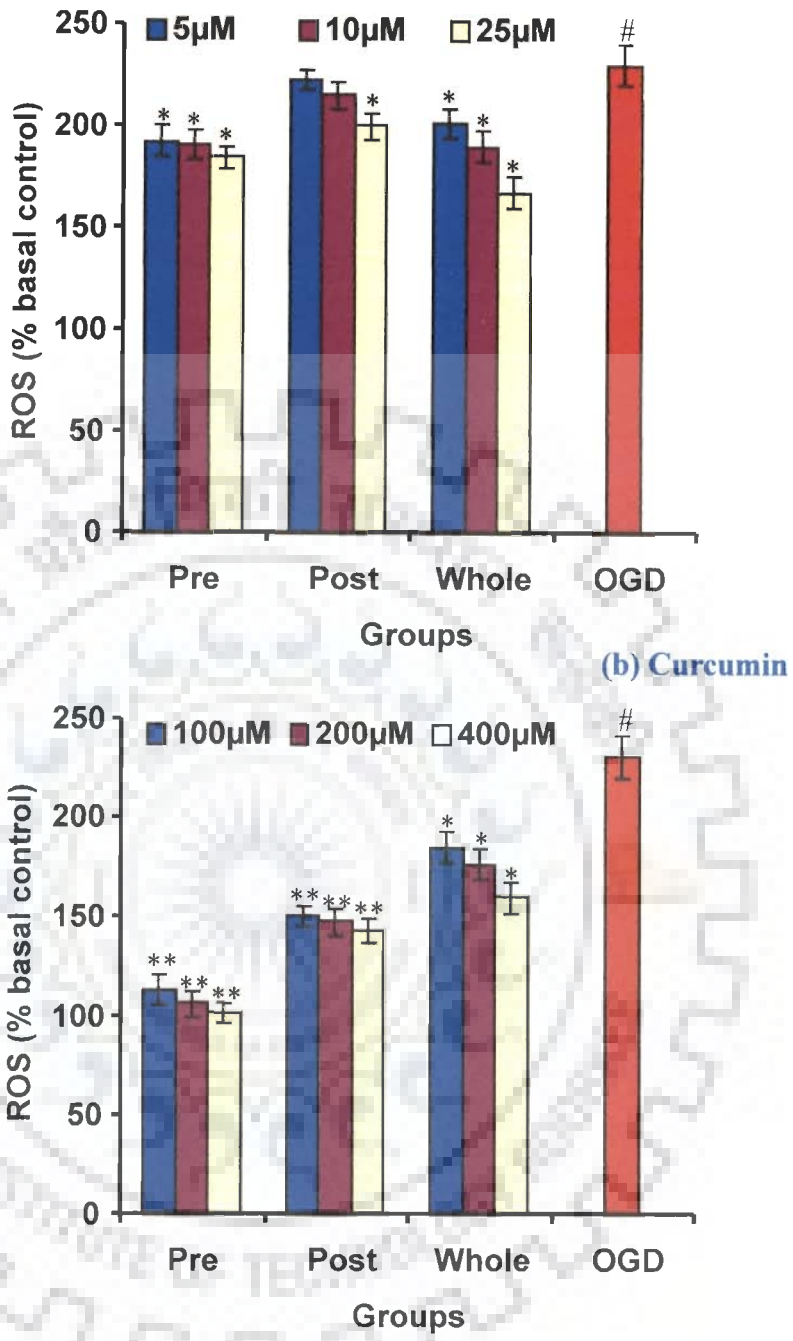


Fig. 4.13a-b: Reactive oxygen species generation in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
*p<0.05, **p<0.01 when compared with OGD

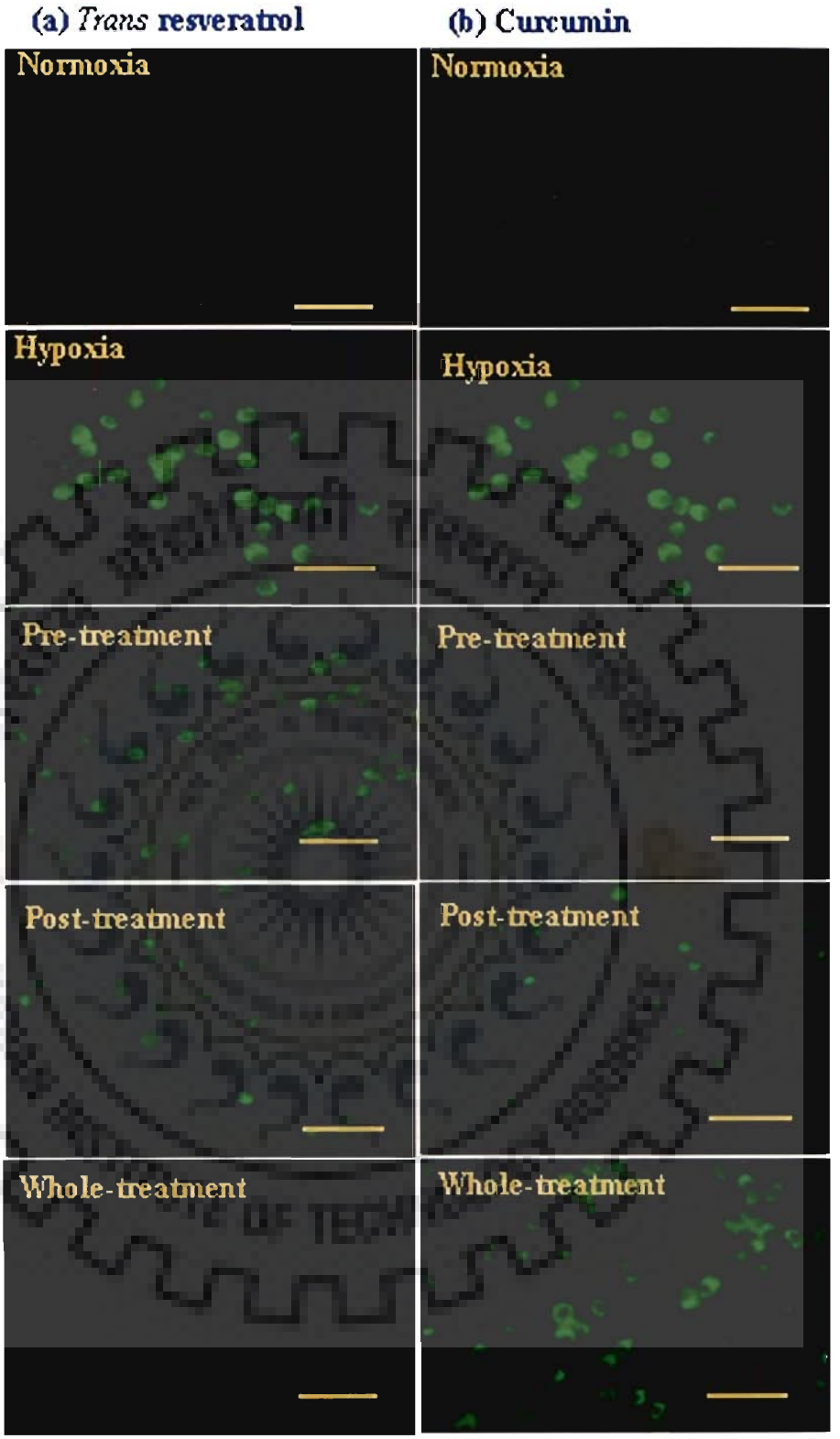


Fig.4.13c OGD-reoxygenation insult induced generation of reactive oxygen species in PC12 cells and restoration with experimental exposure of curcumin and *trans* resveratrol

Images were observed under upright fluorescence microscope (80i Nikon, Japan)

OGD = 6h, reoxygenation= 24 h,

Original magnification = 400x

Scale bar = 100 µm

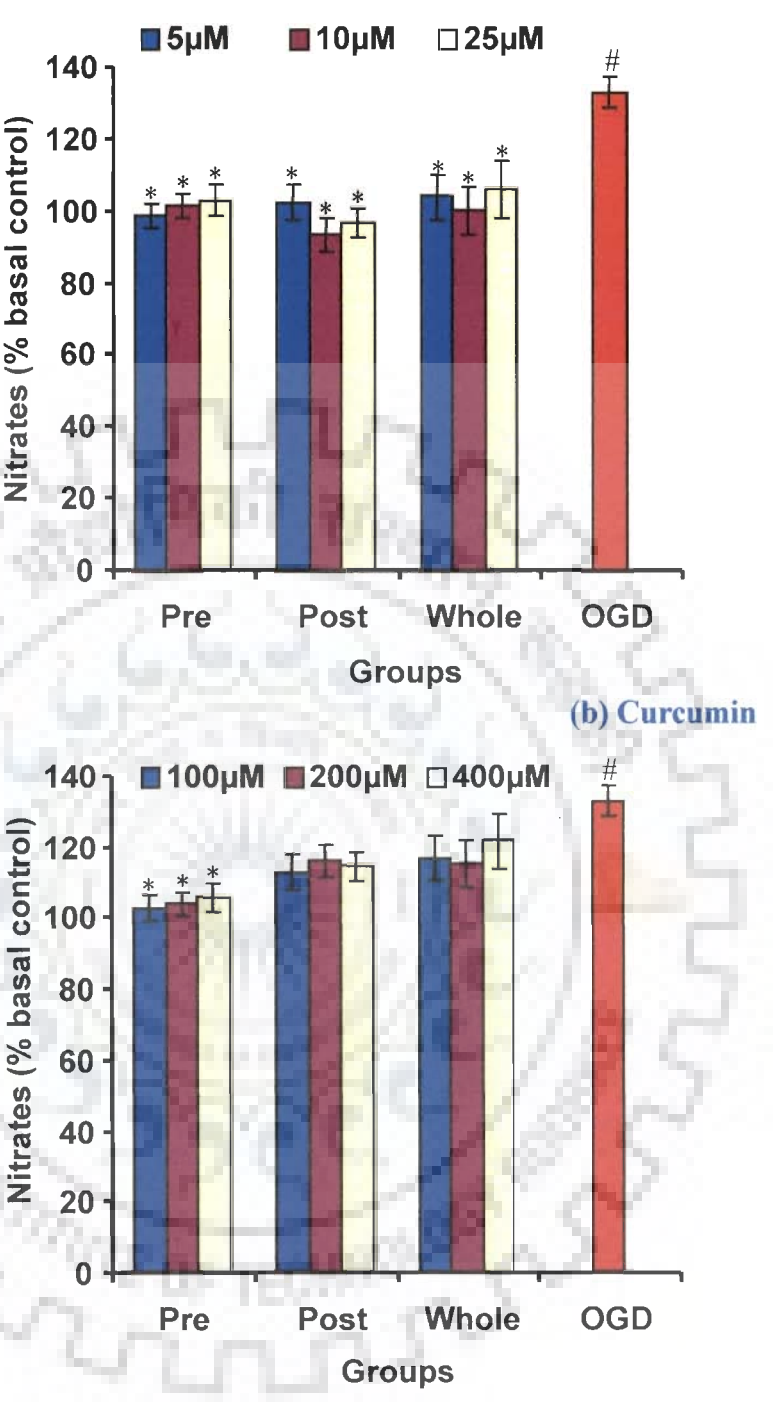


Fig. 4.14a-b: Nitric oxide generation in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
*p<0.05 when compared with OGD

resveratrol showed a concentration dependent significant increase in DA-D_2 receptors i.e. 79.0±3.2, 86.8±4.1 and 93.2±5.4% of normoxia control following the treatment of 5, 10 and 25 μM concentrations respectively (Fig. 4.15a). No increase in receptor concentration was observed in the post and whole-treatment groups of *trans* resveratrol except with 25 μM in post-treatment group (79.5±5.6% of normoxia control).

In case of curcumin also, effects similar to *trans* resveratrol were observed, however the magnitude of recovery was higher and reached very near to normoxia control i.e., 96.0±6.3% at 400 μM concentration of pre-treatment group. In general, pre-treatment both the drugs used in the study were found to be significantly effective for inducing increase in DA-D_2 receptor levels (Fig. 4.15b).

Expression changes at transcriptional (mRNA) level

Transcriptional studies were carried out by semi-quantitative (reverse transcriptase-PCR) and quantitative real time-PCR (RT-PCR^q) methods. Results of OGD-reoxygenation induced alteration in mRNA expression (semi-quantitative RT-PCR) for genes involved in apoptotic and anti-apoptotic pathways and protective potential of *trans* resveratrol and curcumin are summarized in Fig. 4.17-4.28a-b. HPRT mRNA expression in PC12 cells kept under identical experimental conditions was used as internal control to calculate the exposure induced alterations in the mRNA of genes studied (Fig. 4.16a-b).

Reverse Transcriptase semi-quantitative (RT-PCR)

c-Jun: OGD-reoxygenation induced mRNA expression changes for c-Jun gene and modulation capability of *trans* resveratrol and curcumin were studied using specific primers. Statistically highly significant increase in the mRNA expression of c-Jun could be recorded in OGD group i.e., 394.4±14.3% of normoxia control. A statistically significant decrease in the expression of c-Jun was observed in all the treatment schedules of *trans* resveratrol with best performance of pre-treatment (25 μM) group where maximum reduction in mRNA expression was very near to normoxia control i.e., 109.5±10.3% (Fig. 4.17a). A similar pattern of significant reduction in the mRNA expression for c-Jun could be seen following the exposure of curcumin. However, the magnitude of reduction was comparatively higher than *trans* resveratrol and pre-treatment group was found to be best among all the treatment groups (Fig. 4.17b).

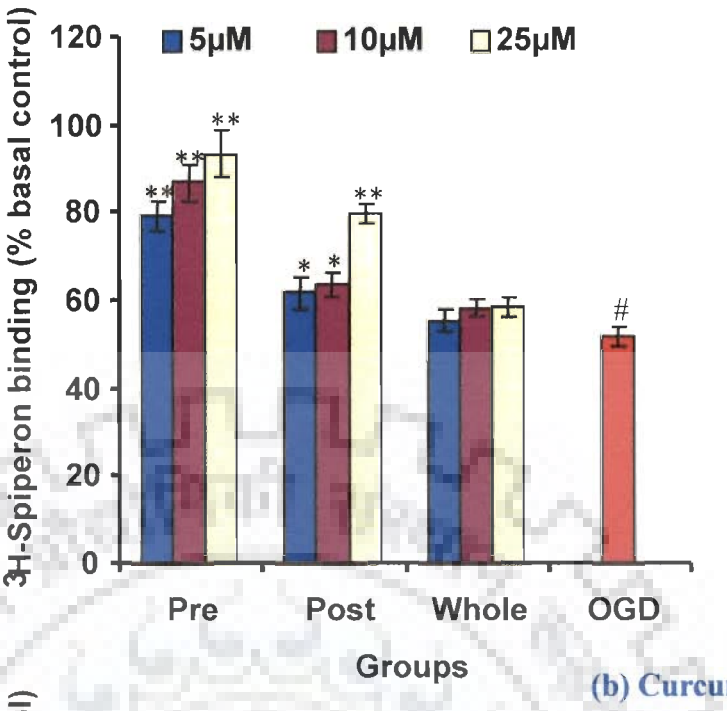
c-Fos: Statistically highly significant increase in the expression of c-fos could be recorded in OGD group i.e., 371.4±16.2% of normoxia control. Percent change in the expression of mRNA for c-Fos was statistically highly significant in whole-treatment group of *trans* resveratrol when compared with OGD as well as normoxia group. The most effective concentration i.e. 25 μM of whole-treatment could bring the values to 108.5±11.3% of normoxia control (Fig. 4.18a). Like c-Jun, in case of c-Fos, pre-treatment with curcumin was found to be most effective in reducing the levels of mRNA in PC12 cells. Curcumin treatment was found to be more effective than *trans* resveratrol in general (Fig. 4.18b).

Bcl-2: OGD-reoxygenation insult was found to cause a significant reduction in the mRNA expression of Bcl-2 in PC12 cells (57.8±5.7% of normoxia control). However, treatment specific changes could be observed when cells were exposed to *trans* resveratrol. Whole-treatment of *trans* resveratrol at 25 μM concentration could bring the values near to the normoxia control (96.7±7.5%) (Fig. 4.19a). Response of curcumin was also similar to *trans* resveratrol, however, like other genes, response of Bcl-2 was seen also with pre-treatment of curcumin (Fig. 4.19b).

Bax and GAP-43: Prominent OGD-reoxygenation induced changes were observed for mRNA expressions for Bax and GAP-43 in PC12 cells (318.3±14.7% and 322.4±19.7% of normoxia control respectively). Interestingly, pre-treatment, in general, was found to be effective for both the drugs at all the concentrations used in the study and was able to bring down the levels of mRNA for Bax and GAP-43 significantly to near normoxia under the experimental conditions (Fig. 4.20-4.21a-b).

Dopamine (DA-D_2) receptor: To further strengthen the data of DA-D_2 receptor binding through radioligand method, studies were also conducted to analyze the OGD induced alterations in the expression of mRNA for DA-D_2 receptor by RT-PCR analysis. With agreement to receptor binding results RT-PCR analysis also showed a significant reduction in the mRNA expression (48.6±6.2% of normoxia control) in DA-D_2 receptors in cells of OGD group. A concentration-dependent increase in the expression could be seen in whole-treatment group of *trans* resveratrol. No remarkable response in pre or post-treatment groups could be seen, except in case of 100, 25 μM dose of *trans* resveratrol (Fig. 4.22a). For curcumin, the trend of expressional changes was similar to *trans* resveratrol, however the magnitude of recovery was

(a) *Trans* resveratrol



(b) Curcumin

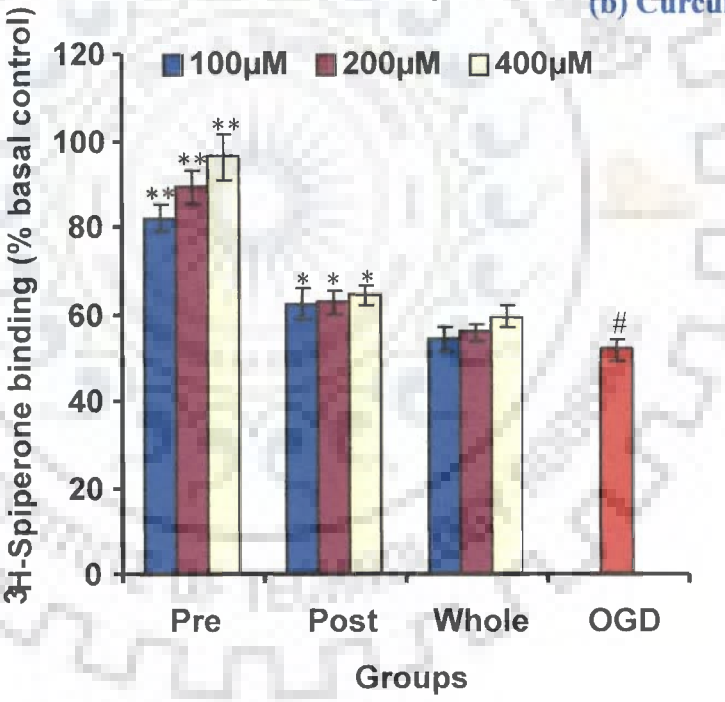


Fig. 4.15a-b: Dopamine DA-D_2 receptor levels in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

#p<0.05 when OGD compared with basal control
*p<0.05, **p<0.01 when compared with OGD

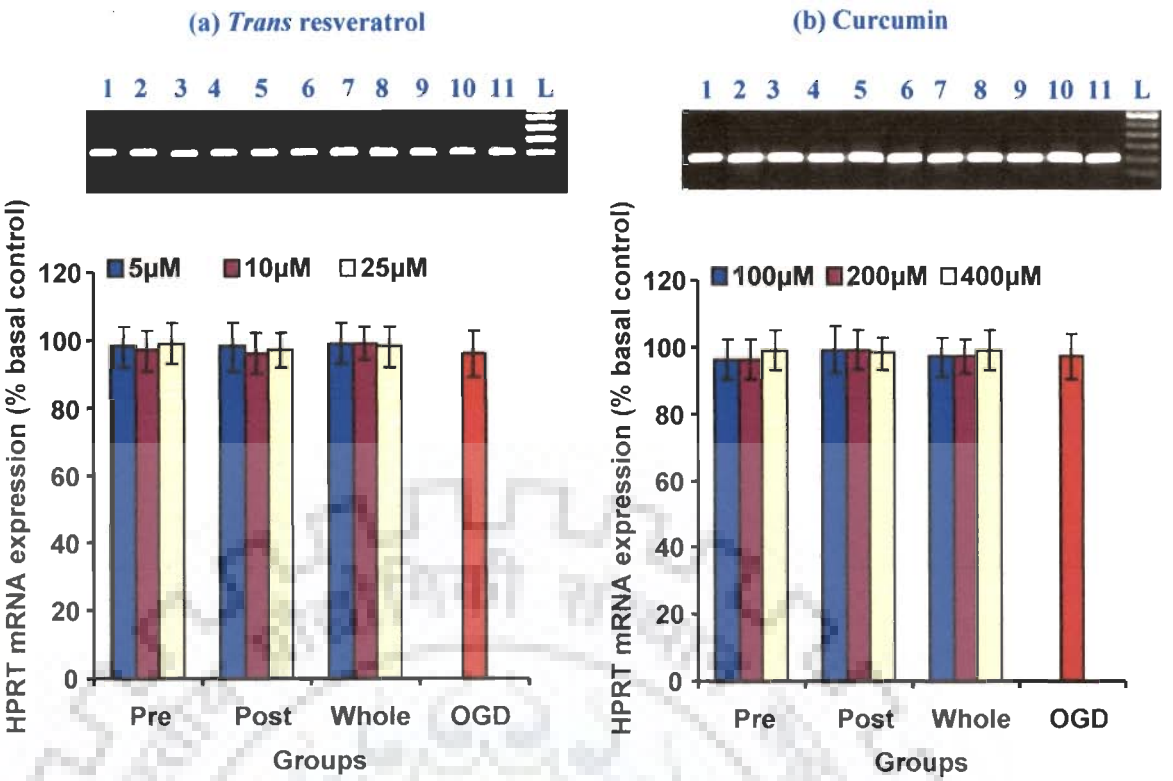


Fig. 4.16a-b: mRNA expression for HPRT in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

Fig.a:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control. Fig.b:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; L: Ladder DNA marker.

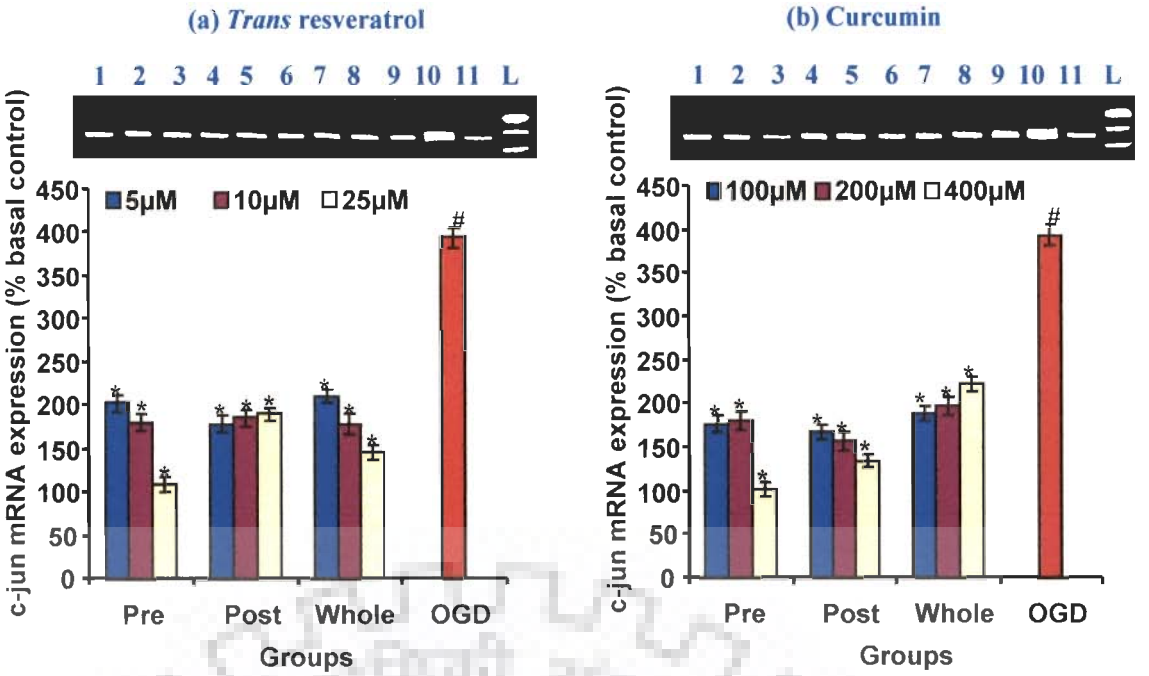


Fig. 4.17a-b: mRNA expression for c-jun in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

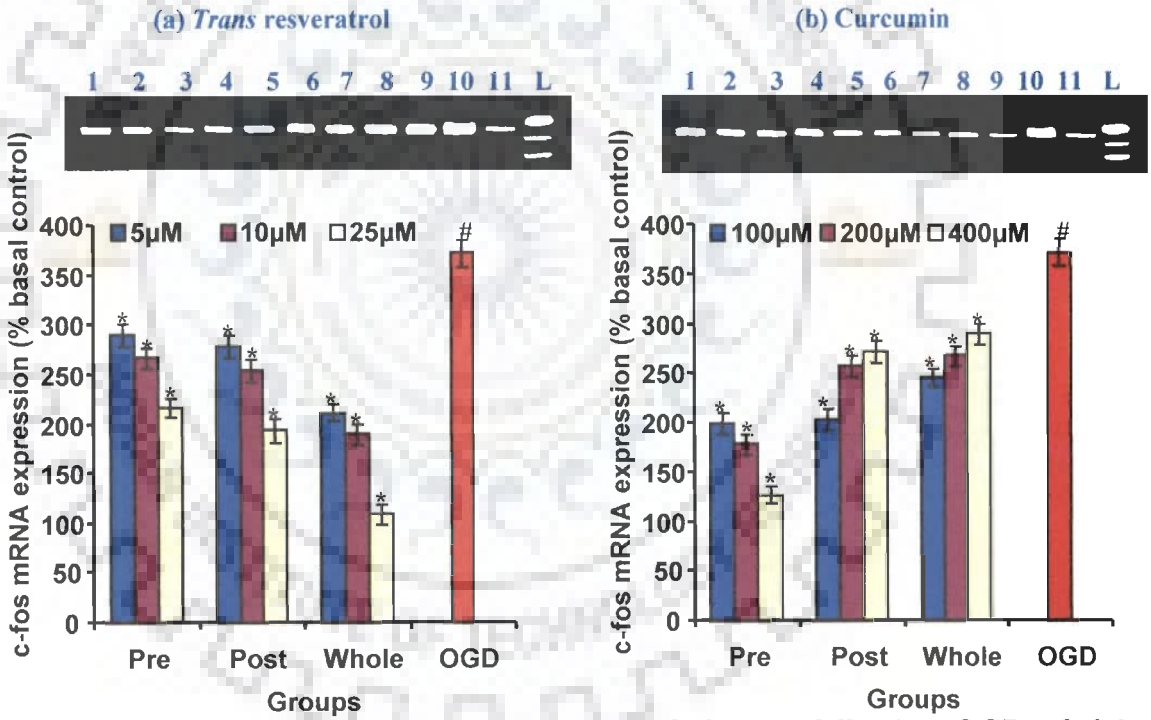


Fig. 4.18a-b: mRNA expression for c-fos in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

Fig.a:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control. Fig.b:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; L: Ladder DNA marker; #$p<0.05$ when OGD compared with basal control. *$p<0.05$, **$p<0.01$ when compared with OGD

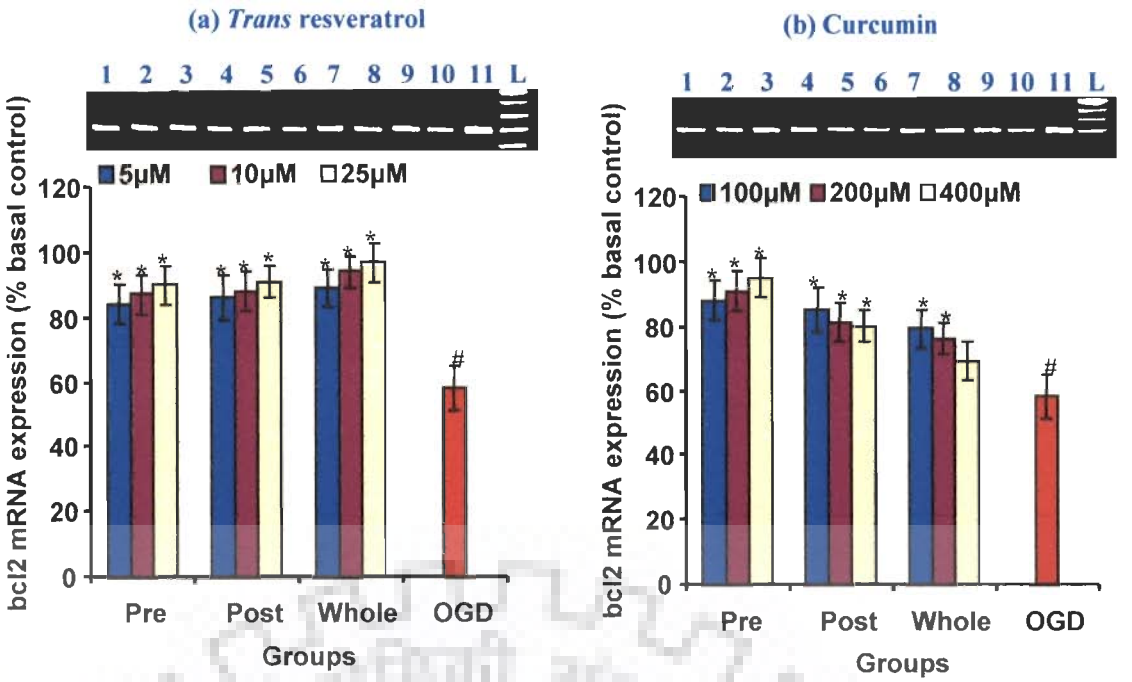


Fig. 4.19a-b: mRNA expression for bcl-2 in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

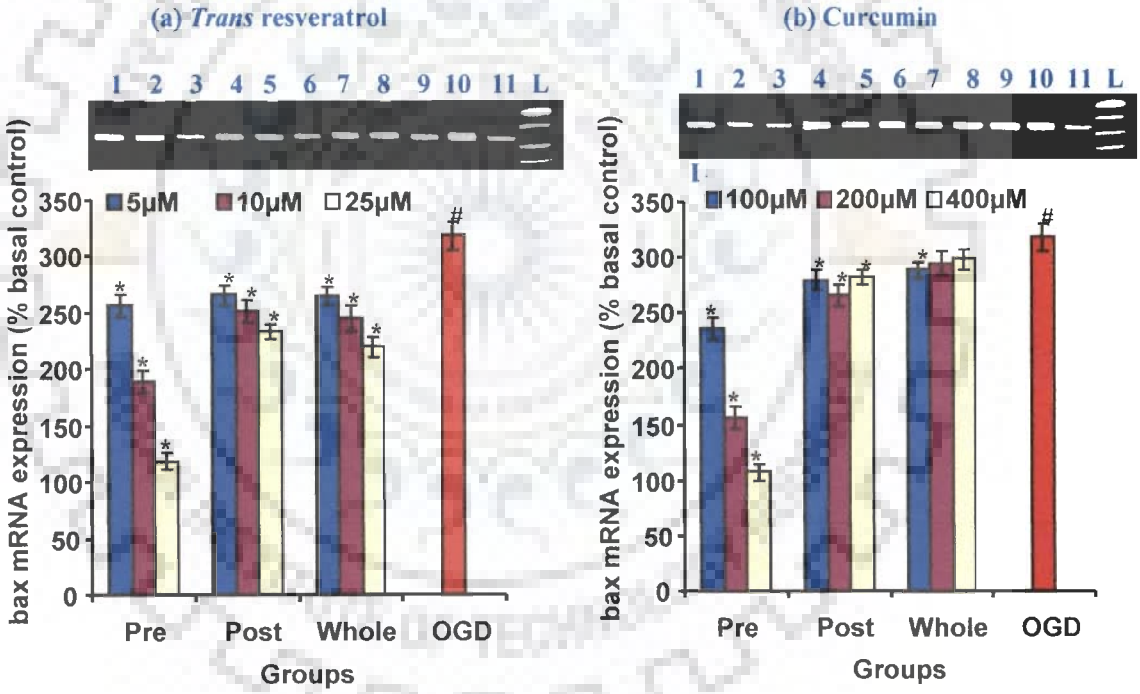


Fig. 4.20a-b: mRNA expression for bax in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

Fig.a:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control. Fig.b:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; L: Ladder DNA marker; #$p<0.05$ when OGD compared with basal control. *$p<0.05$, **$p<0.01$ when compared with OGD

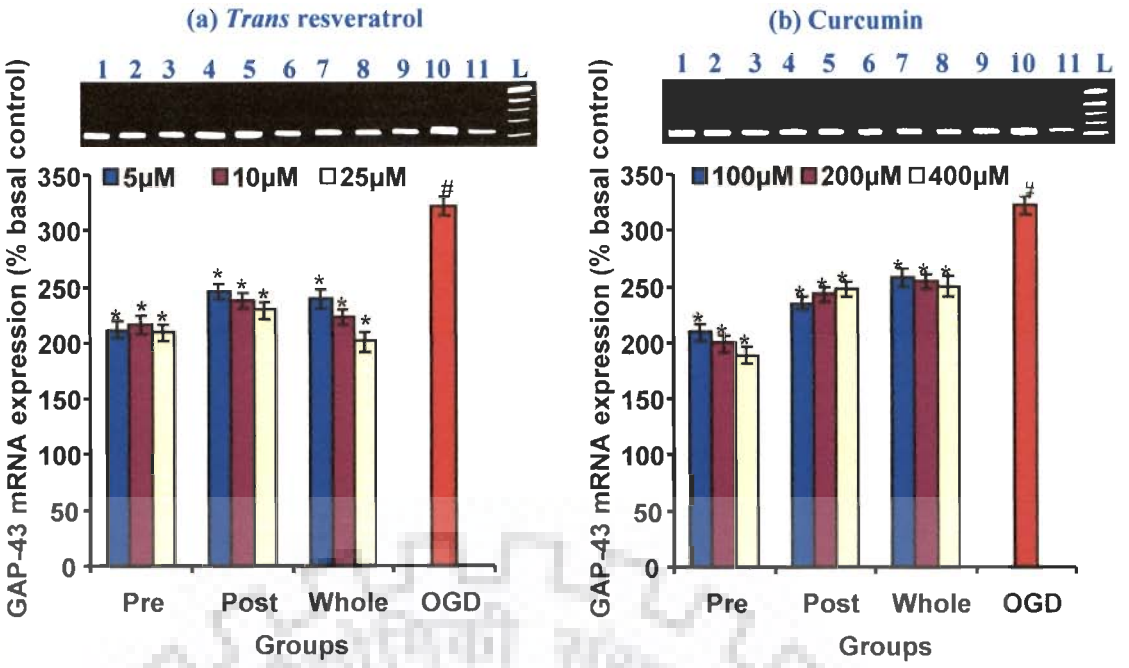


Fig. 4.21a-b: mRNA expression for GAP-43 in PC12 cells following OGD of 6h and reoxygenation of 24 h and affect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

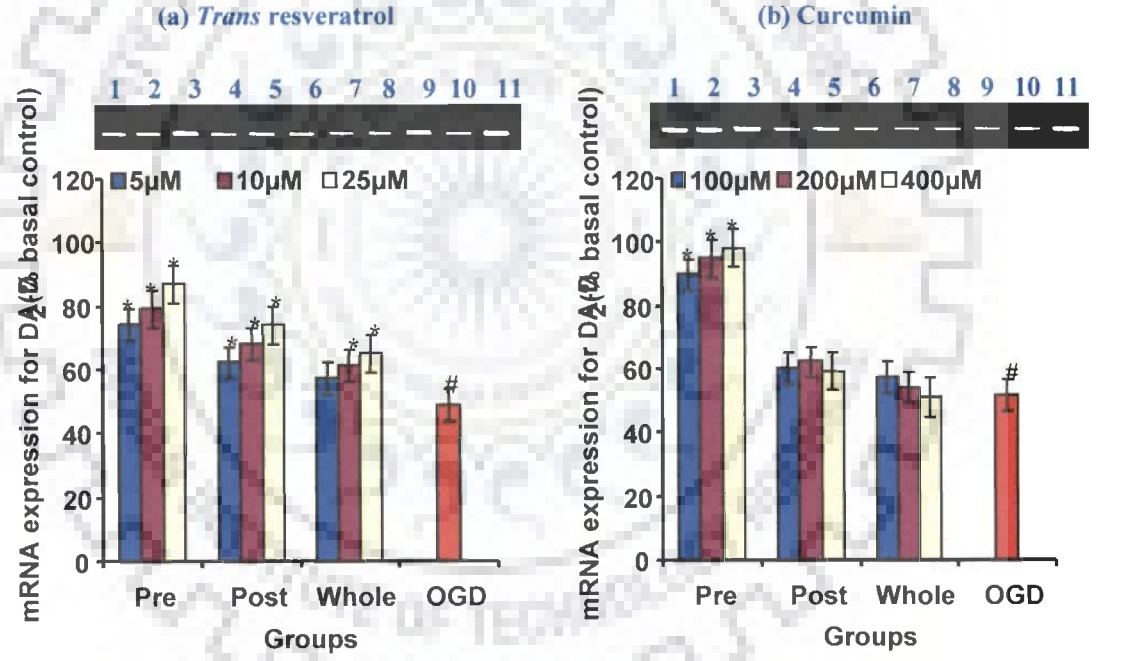


Fig. 4.22a-b: mRNA expression for DA-D_2 in PC12 cells following OGD of 6 h and reoxygenation of 24 h and affect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

Fig.a:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control. Fig.b:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; L: Ladder DNA marker; #$p<0.05$ when OGD compared with basal control. *$p<0.05$, **$p<0.01$ when compared with OGD

higher in pre-treatment group i.e. 89.8±5.2%, 95.0±4.8% and 98.0%±7.1% of normoxia control following the treatment of 100, 200 and 400 μM respectively (Fig. 4.22b).

Real Time Quantitative (RT-PCR^q)

BIM: Significant rise in the m-RNA expression of BIM was recorded in OGD group i.e. ≈ 3.1 fold of normoxia control. Furthermore, significant increase in BIM m-RNA was also recorded in both 6 and 24 h post hypoxic/reoxygenation groups (5.5 and 2.4 fold respectively). Statistically significant decrease in the expression of BIM m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of whole treatment group, where maximum 2 fold reduction in the mRNA expression was observed in 24 h whole treatment group when compared to OGD only (Fig. 4.23a). Concomitantly, non-significant pattern of decrease was also recorded in both the immediate and 6 h groups (0.7 and 0.6 fold respectively) when compared to hypoxic group only.

Cav-β3: Statistically significant rise in the m-RNA expression of Cav-β3 was recorded in OGD group i.e. ≈ 1.4 fold of normoxia control. Furthermore, significant increase in Cav-β3 m-RNA was also recorded in 6 h post hypoxic/reoxygenation group (3.2 fold), however significant decrease was observed in 24 h post hypoxic/ reoxygenation group (0.3 fold). Statistically significant decrease in the expression of Cav-β3 m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of whole treatment group, where maximum 1.2 fold reduction in the mRNA expression was observed in 24h whole treatment group when compared to OGD only (Fig. 4.23b). Concomitantly, non-significant pattern of decrease was recorded in immediate whole treatment group (0.4 fold), however significant increase was observed in 6h whole treatment group (1 fold), when compared to hypoxic group only.

p21: Statistically higher and significant rise in the m-RNA expression of p21 was recorded in OGD group i.e. ≈ 2.6 fold of normoxia control. Furthermore, significant increase in p21 m-RNA was also recorded in 6 h post hypoxic/reoxygenation group (3.3 fold), however significant decrease was observed in 24 h post hypoxic/ reoxygenation group (0.5 fold). Statistically significant decrease in the expression of p21 m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of whole treatment group, where maximum 2.3 fold reduction in the

mRNA expression was observed in 24 h whole treatment group when compared to OGD only (Fig. 4.24a). Concomitantly, non significant pattern of decrease was recorded in immediate and 6 h whole treatment group (0.6 and 0.2 fold respectively), when compared to hypoxic group only.

MMP11: Significant rise in the m-RNA expression of MMP11 was recorded in OGD group i.e. ≈ 2.3 fold of normoxia control. Furthermore significant increase in MMP11 m-RNA was also recorded in both 6 and 24 h post hypoxic/reoxygenation groups (4.7 and 7.6 fold respectively). Statistically significant decrease in the expression of MMP11 m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of whole treatment group, where maximum 0.8 fold reduction in the mRNA expression was observed in immediate group when compared to OGD only (Fig. 4.24b). In contrast to the above, significant pattern of increase was recorded in both 6 and 24 h in all treatment groups, when compared to hypoxic group only.

HIF-1α: Significant rise in the m-RNA expression of HIF-1α was recorded in OGD group i.e. ≈ 990 fold of normoxia control. Furthermore, significant increase in HIF-1α m-RNA was also recorded in both the 6 and 24h post hypoxic/reoxygenation groups (750 and 600 fold respectively). Statistically significant decrease in the expression of HIF-1α m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of whole treatment group, where maximum 850 fold reduction in the mRNA expression was observed in 24h whole treatment group when compared to OGD only (Fig. 4.25a). Concomitantly, significant pattern of decrease was also recorded in both the immediate and 6h groups (290 and 190 fold respectively) when compared to hypoxic group only.

GAPDH: Significant rise in the m-RNA expression of GAPDH was recorded in OGD group i.e. ≈ 3.5 folds of normoxia control. Furthermore, significant increase in GAPDH m-RNA was also recorded in both 6 and 24 h post hypoxic/reoxygenation groups (4.3 and 2.4 fold respectively). Statistically significant, decrease in the expression of GAPDH m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of whole treatment group, where maximum 2.4 fold reduction in the mRNA expression was observed in 24 h whole treatment group when compared to OGD only (Fig. 4.25b). Concomitantly, significant pattern of decrease was also recorded in both the immediate and 6h groups (1.3 and 2.1 fold respectively) when compared to hypoxic group only.

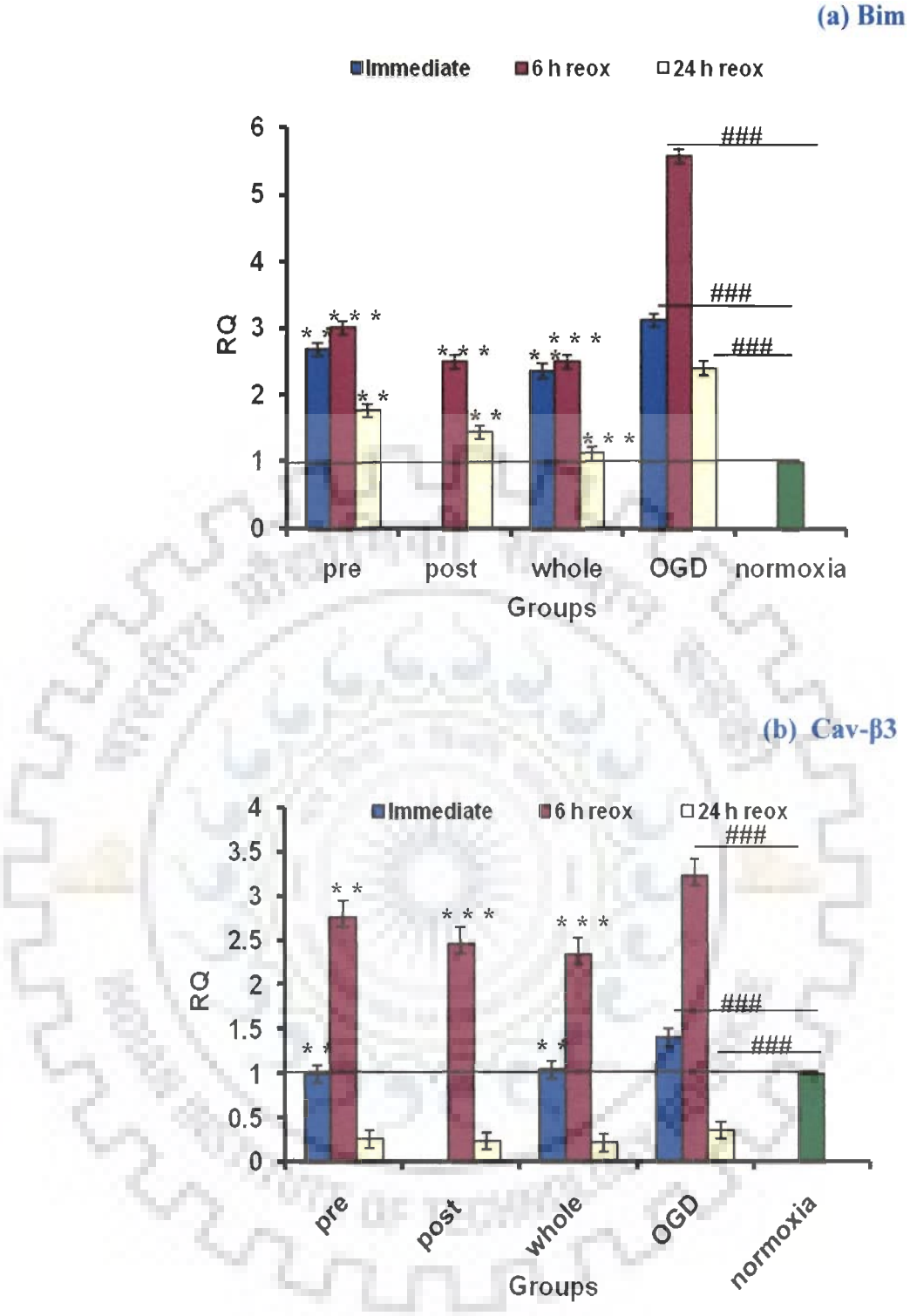


Fig. 4.23a-b: mRNA expression for bim and cavβ3 in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various treatment of *trans* resveratrol

###p<0.001 when OGD compared with normoxia control.
**p<0.01, ***p<0.001 when compared with OGD

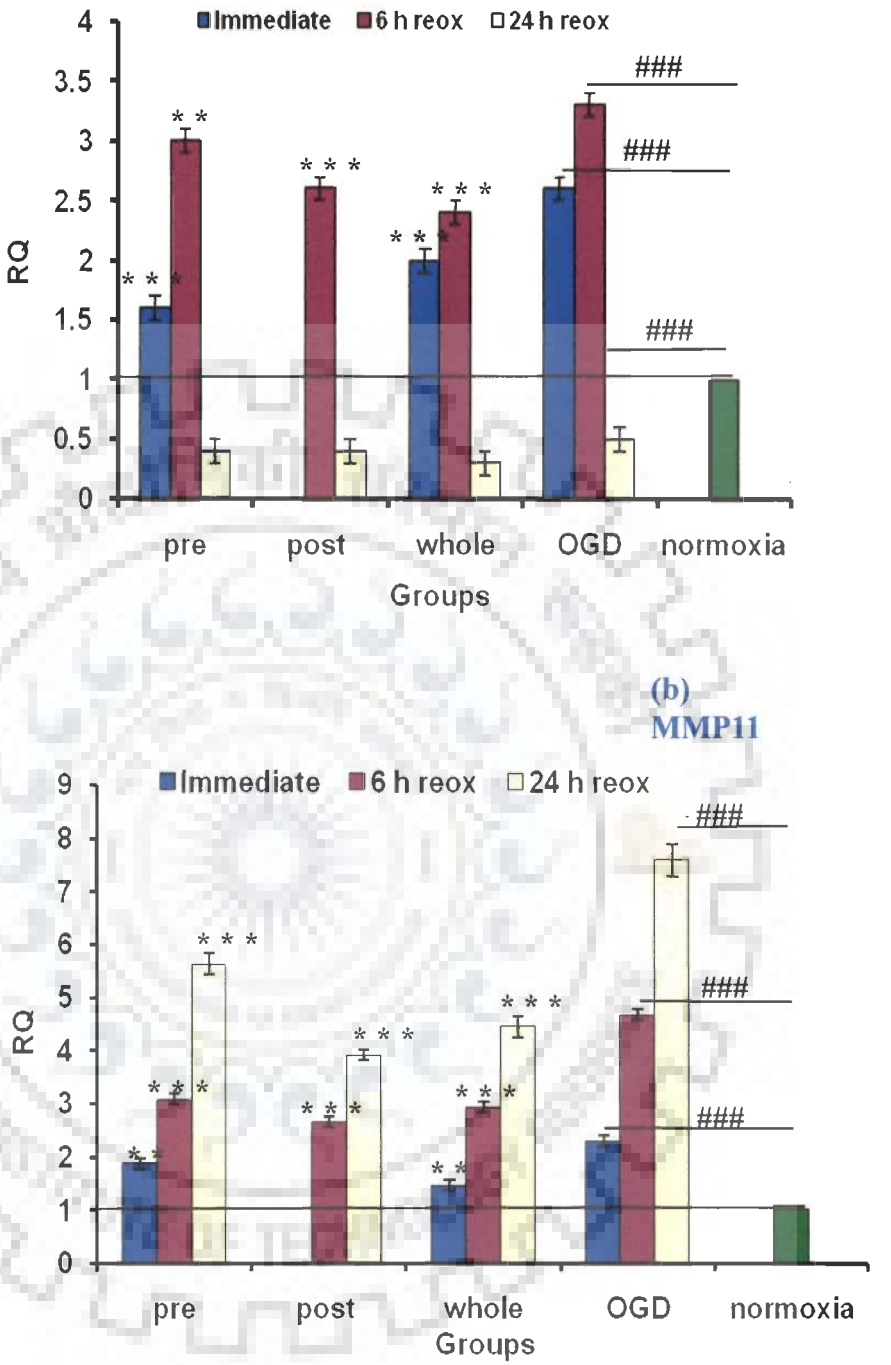


Fig. 4.24a-b: mRNA expression for P21 and MMP11 in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various treatment of *trans* resveratrol

###p<0.001 when OGD compared with normoxia control.
**p<0.01, ***p<0.001 when compared with OGD

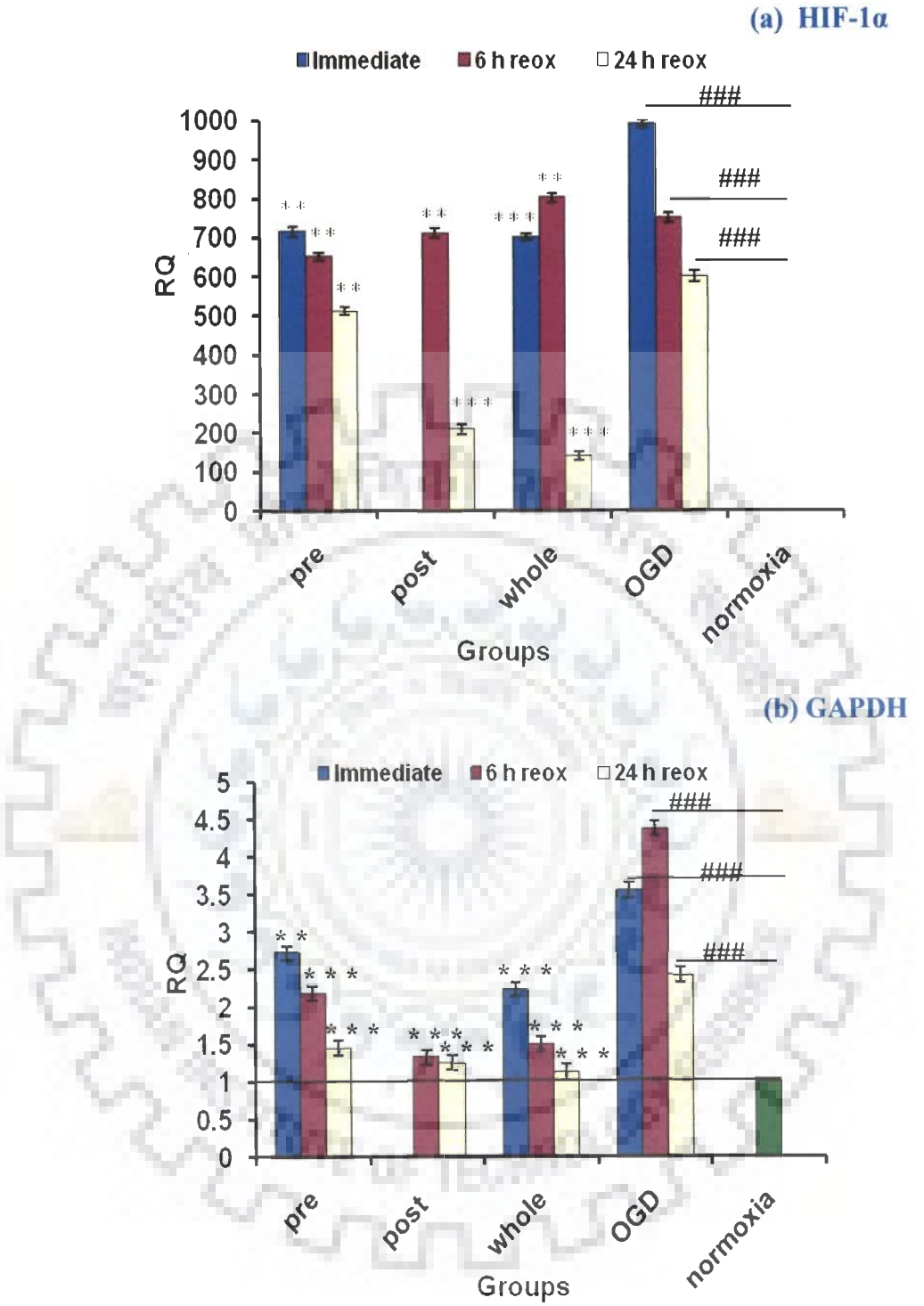


Fig. 4.25a-b: mRNA expression for HIF-1α and GAPDH in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various treatment of *trans* resveratrol

###p<0.001 when OGD compared with normoxia control.
**p<0.01, ***p<0.001 when compared with OGD

STAT3: Significant rise in the m-RNA expression of STAT3 was recorded in OGD group i.e. ≈ 3.1 fold of normoxia control. Furthermore, significant increase in STAT3 m-RNA was also recorded in both 6 and 24 h post hypoxic/reoxygenation groups (3.9 and 2.5 fold respectively). Statistically significant decrease in the expression of STAT3 m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of whole treatment group, where maximum 1.8 fold reduction in the mRNA expression was observed in 24 h whole treatment group when compared to OGD only (Fig. 4.26a). Concomitantly, significant pattern of decrease was also recorded in both the immediate and 6 h whole treatment group (1.4 and 1 fold respectively), when compared to hypoxic group only.

Hsp27: Statistically significant rise in the m-RNA expression of Hsp27 was recorded in OGD group i.e. ≈ 6.9 fold of normoxia control. Furthermore, significant increase in Hsp27 m-RNA was also recorded in 6 h post hypoxic/reoxygenation group (8.9 fold), however significant decrease was observed in 24 h post hypoxic/ reoxygenation group (0.5 fold). Statistically significant decrease in the expression of Hsp 27 m-RNA was observed in all the treatment schedules of *trans* resveratrol except 6 h pre treatment group (1.1 fold increase) when compare to immediate OGD group. Best performance of pre treatment group, where maximum 6.5 fold reduction in the mRNA expression was observed in 24 h pre treatment group when compared to OGD only (Fig. 4.26b). Concomitantly, non-significant pattern of decrease was recorded in immediate pre treatment group (0.9 fold), when compared to hypoxic group only.

PTGER2: Statistically significant rise in the m-RNA expression of PTGER2 was recorded in OGD group i.e. ≈ 2.5 fold of normoxia control. Furthermore, significant increase in PTGER2 m-RNA was also recorded in both 6 and 24 h post hypoxic/reoxygenation groups (3.2 and 2.1 fold respectively). Statistically significant decrease in the expression of PTGER2 m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of pre and whole treatment groups, where maximum 1 fold reduction in the mRNA expression was observed in immediate and 24 h group, when compared to OGD only (Fig.4.27a). Concomitantly, significant pattern of decrease was also recorded in 6 h post hypoxic/ reoxygenation groups (0.7fold)when compared to hypoxic group only.

GSTP1: Statistically significant rise in the m-RNA expression of GSTP1 was recorded in OGD group i.e. ≈ 4.5 fold of normoxia control. Furthermore, significant

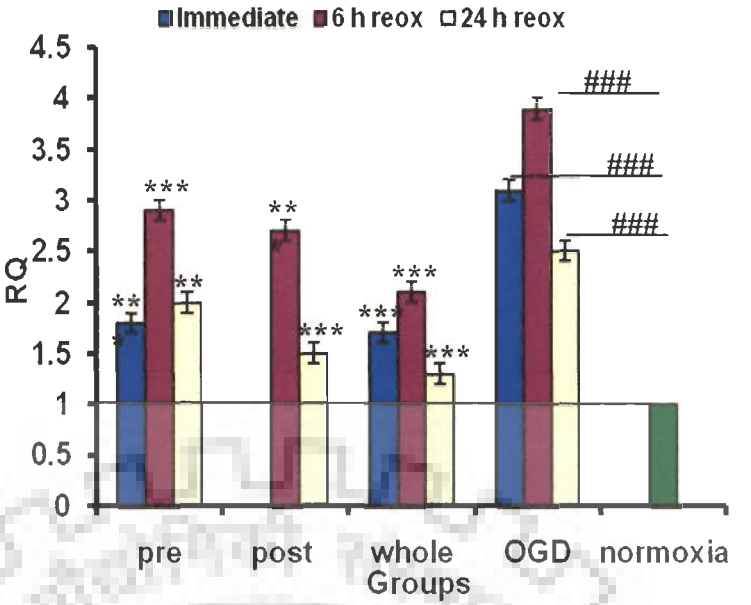
increase in GSTP1 m-RNA was also recorded in both 6 and 24 h post hypoxic/reoxygenation groups (7.8 and 2.5 fold respectively). Statistically significant decrease in the expression of GSTP1 m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of whole treatment group, where maximum 3.5 fold reduction in the mRNA expression was observed in 24 h whole treatment group when compared to OGD only (Fig. 4.27b). Concomitantly, significant pattern of decrease was also recorded in both the immediate and 6 h groups (2 and 1 fold respectively) when compared to hypoxic group only.

Bax: Statistically higher and significant rise in the m-RNA expression of Bax was recorded in OGD group i.e. ≈ 4 fold of normoxia control. Furthermore, significant increase in Bax m-RNA was also recorded in both 6 and 24h post hypoxic/reoxygenation groups (6.1 and 2.8 fold respectively). Statistically significant decrease in the expression of Bax m-RNA was observed in all the treatment schedules of *trans* resveratrol with best performance of whole treatment group, where maximum 3 fold reduction in the mRNA expression was observed in 24 h whole treatment group when compared to OGD only (Fig. 4.28a). Concomitantly, significant pattern of decrease was also recorded in both the immediate and 6h groups (1.3 and 2.2 fold respectively) when compared to hypoxic group only.

Expression studies

Attempts were made to study the status of genes involved in immediate early responses and apoptotic pathways involved in OGD-reoxygenation stress and the restoration of them, if any, following the treatment of *trans* resveratrol and curcumin at translational levels. In western blot analysis, HPRT was kept as internal control to calculate the expressional changes following OGD insult and reoxygenation and restoration with *trans* resveratrol and curcumin treatments (Fig. 4.30a-b). The result highlights of protein expression studies using both immunocytochemistry and western blotting are presented in Fig. 4.29a-b, 4.31-36a-d.

Status of 4-hydroxynonenal (HNE): HNE is a membrane derived aldehyde which has been found to be involved in the pathophysiology of cerebral ischemia. Expressional changes at protein levels following OGD-reoxygenation and groups treated with *trans* resveratrol and curcumin were studied using immunocytochemical localization method with the help of specific monoclonal antibody. After live cell



(b) Hsp27

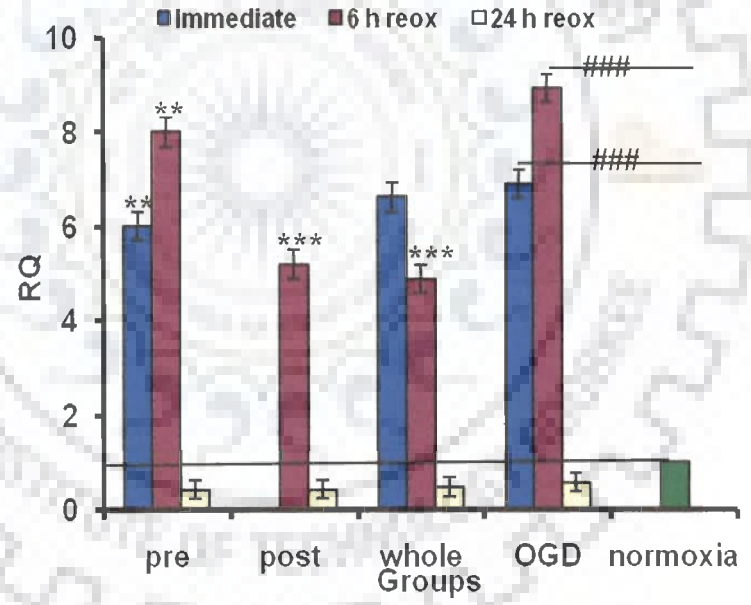
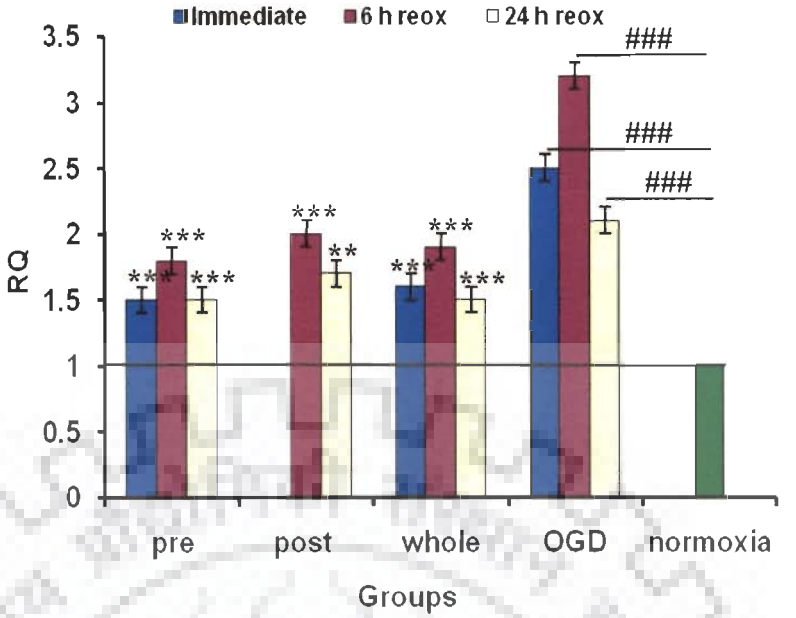


Fig. 4.26a-b: mRNA expression for STAT3 and Hsp27 in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various treatment of *trans* resveratrol

###p<0.001 when OGD compared with normoxia control.
**p<0.01, ***p<0.001 when compared with OGD

(a)PTGER2



(b) GSTP1

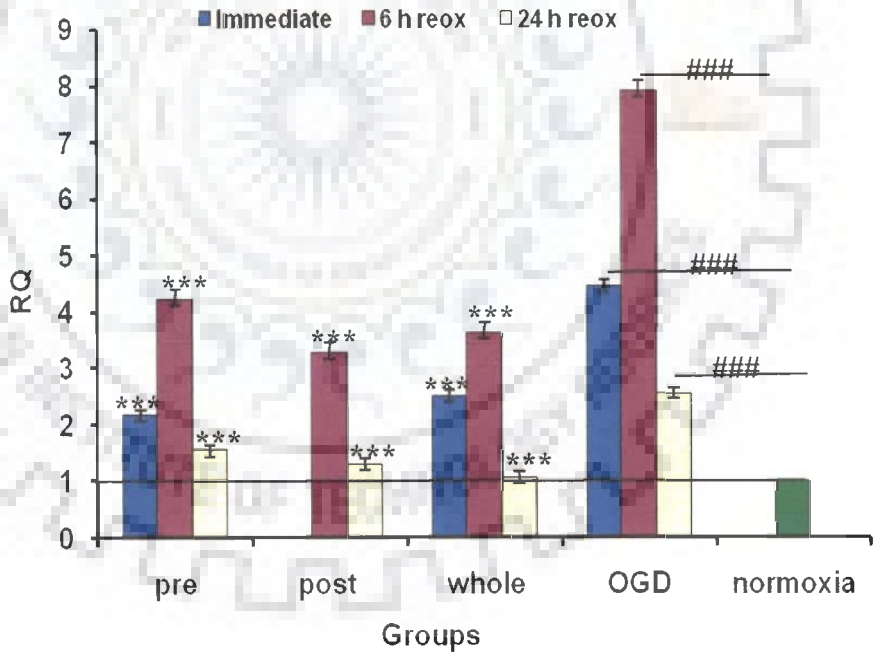


Fig. 4.27a-b: mRNA expression for PTGER2 and GSTP1 in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various treatment of *trans* resveratrol

###p<0.001 when OGD compared with normoxia control.
**p<0.01, ***p<0.001 when compared with OGD

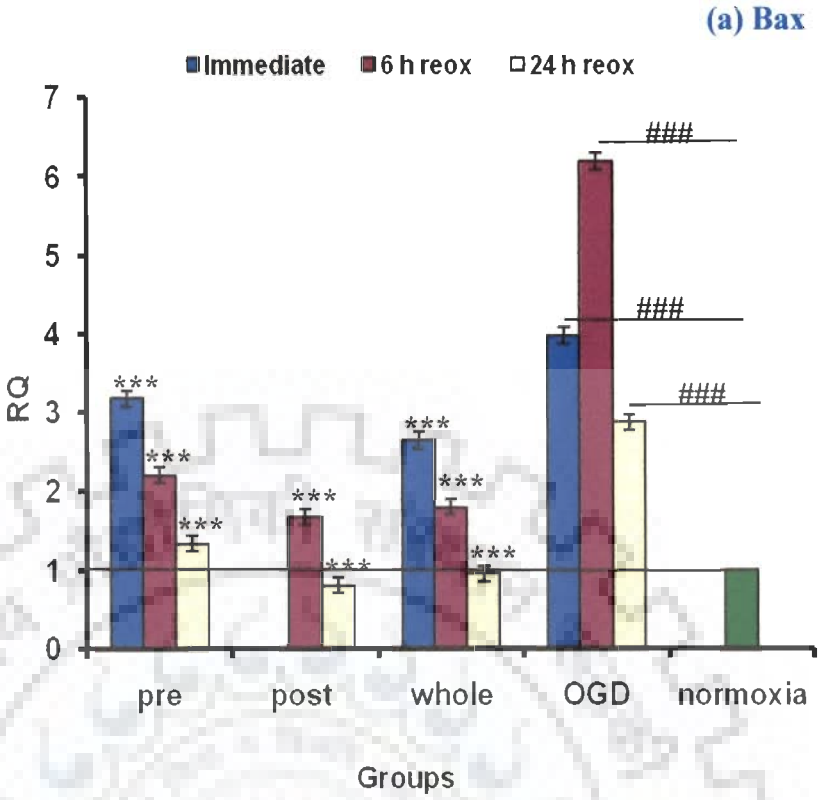


Fig. 4.28: **mRNA expression for Bax in PC12 cells following OGD of 6 h and reoxy genation of 24 h and effect of various treatment of *trans* resveratrol**

###p<0.001 when OGD compared with normoxia control.
**p<0.01, ***p<0.001 when compared with OGD

imaging, fluorescence quantification was done to analyze the alteration in the expression of intracellular protein. Significant increase in the production of HNE could be recorded in OGD group i.e. 235.1±16.3% of normoxia control. All the treatment schedules of *trans* resveratrol exhibited protective potential to some extent with maximum response in whole-treatment group; however, the values were higher than normoxia control group (Fig. 4.29a).

Response of curcumin was found to be more or less similar to *trans* resveratrol, however the maximum effect could be detected in 100 and 200 μM concentrations used in pre-treatment group i.e. 162.2±11.4, 151.4±9.3% of normoxia control (Fig. 4.29b). Overall, response of both the drugs used on the restoration of OGD-reoxygenation induced changes was considered non-significant.

Immunocytochemical quantification of c-Jun protein: OGD-reoxygenation induced expressional changes for c-Jun gene protein and modulation capability of *trans* resveratrol and curcumin were studied using specific monoclonal antibody. Statistically significant increase in the expression of c-Jun could be recorded in OGD group i.e., 204.4±12.3% of normoxia control. A statistically significant decrease in the expression of c-Jun was observed in all the treatment schedules of *trans* resveratrol, but none of the concentration was found capable of bringing back the values to basal levels i.e. up to normoxia control. *Trans* resveratrol concentration (25 μM) showed maximum reduction in all the treatment groups i.e. pre (139.7±9.6%), post (172.5±12.3%) and whole-treatment (175.6±11.5%) groups. However, the overall reduction was fairly higher than the normoxia control (Fig. 4.31a).

Minor reductions in the expression of c-Jun protein could be seen following exposure of curcumin. The magnitude of reduction was not significant and less prominent than that observed with *trans* resveratrol (Fig. 4.31b). In general, curcumin was not found effective to restore the expression changes of c-Jun in OGD received PC12 cells.

Western blot analysis for c-Jun protein: In western blot analysis, similar trend was observed for c-Jun protein expression but the values were on the lower side. Dendographic analysis shows a significant increase in c-Jun protein in OGD group i.e. 192.1±10.9% of normoxia control. Although, significant reduction in the expression of c-Jun protein could be recorded following *trans* resveratrol treatment, but none of the

treatment schedule was found capable to bring back the values to basal levels i.e. up to normoxia control. *Trans* resveratrol at 25 μM concentration could pose maximum reduction in all the treatment groups i.e. pre (133.4±8.9%), post (129±7.6%) and whole (128±7.9%). However, the overall reduction was fairly higher than the normoxia control (Fig. 4.31c). In western blot analysis, no significant reduction in the expression of c-Jun protein following curcumin treatment at any concentration could be detected (Fig. 4.31d).

Immunocytochemical quantification of c-Fos protein: Statistically high significant increase in the expression of c-Fos could be recorded in OGD group i.e., 196.4±14.8% of normoxia control. Percent change in the expression of c-Fos was significant in whole-treatment group of *trans* resveratrol when compared with OGD group but was insignificant as compared to normoxia control. The most effective concentration of *trans* resveratrol i.e. 25μM of whole-treatment group could only bring the values to 131.5±9.3% of normoxia control (Fig. 4.32a).

Like c-Jun, in case of c-Fos, none of the curcumin treatment schedules could significantly reduce the protein expression. Curcumin exposure was also found to be less effective than *trans* resveratrol (Fig. 4.32b).

Western blot analysis for c-Fos protein: Results of western blot analysis for the expression of c-Fos gene protein are in agreement with results obtained with immunocytochemistry. OGD group was found to induce the expression approximately 2 fold i.e. 188.9±9.3% of normoxia control. Though, a reduction in expression following *trans* resveratrol was observed but it was insignificant except in the whole-treatment group at 25 μM concentration (Fig. 4.32c). Once again, curcumin treatment was found to be ineffective in reducing the expression of c-Fos protein (Fig. 4.32d).

Immunocytochemical quantification of Bcl-2 protein: OGD insult was found to cause a significant reduction in the expression of Bcl-2 protein in PC12 cells (78.3±7.7% of normoxia control). No treatment specific changes could be observed when cells were exposed to *trans* resveratrol, however drug treatment enhanced the expression of Bcl-2 (Fig. 4.33a).

Response of curcumin was also similar to *trans* resveratrol except in case of whole-treatment group where no improvement was observed. Instead, a further

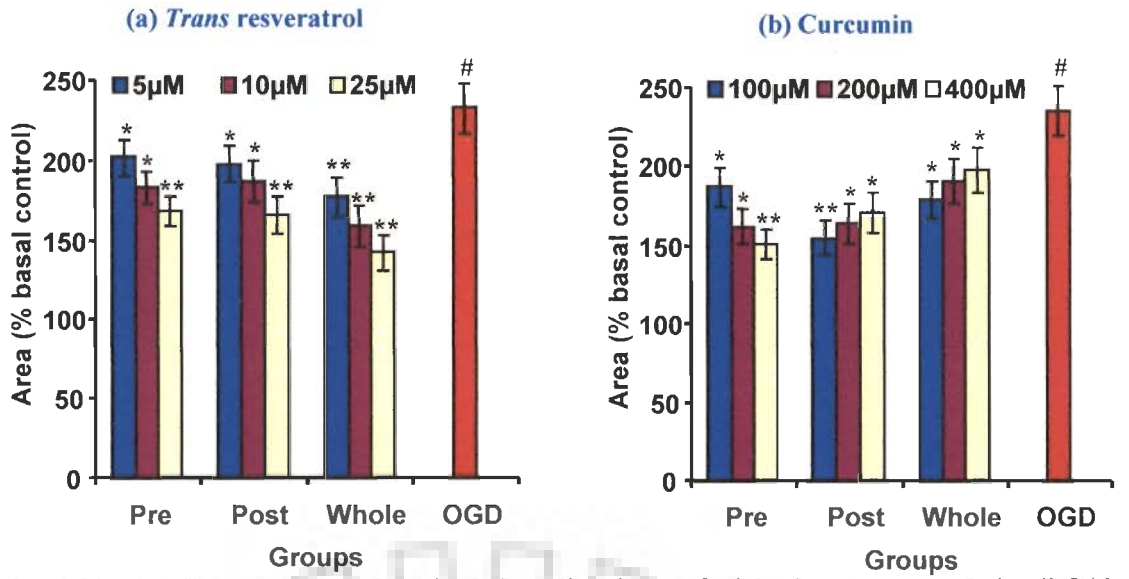


Fig. 4.29a-b: Immunocytochemical localization of 4-hydroxynonenal in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

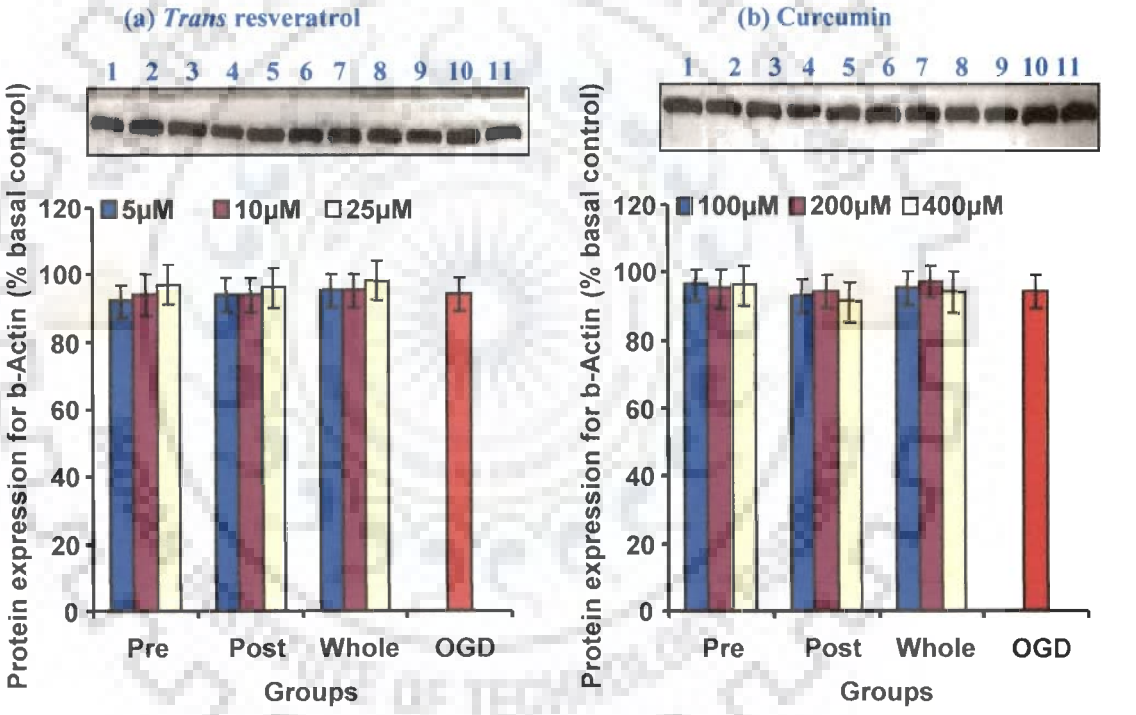


Fig. 4.30a-b: Protein expression for HPRT using western blot analysis in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (c) and curcumin (d) treatment

Fig.a:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control. Fig.b:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; #$p<0.05$ when OGD compared with basal control. *$p<0.05$, **$p<0.01$ when compared with OGD

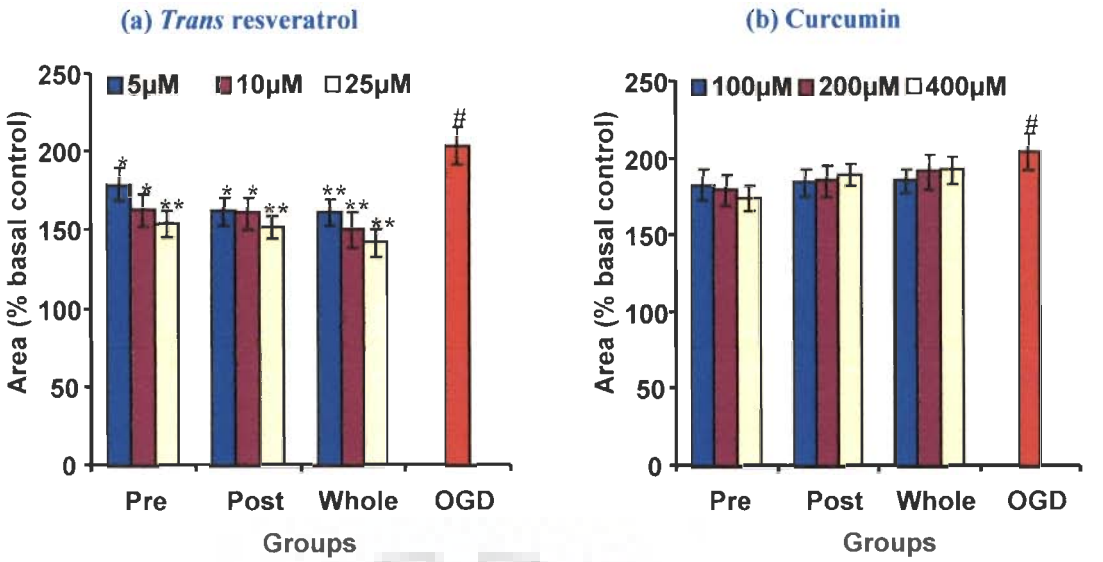


Fig. 4.31a-b: Expression of c-Jun gene protein using immunocytochemical technique in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

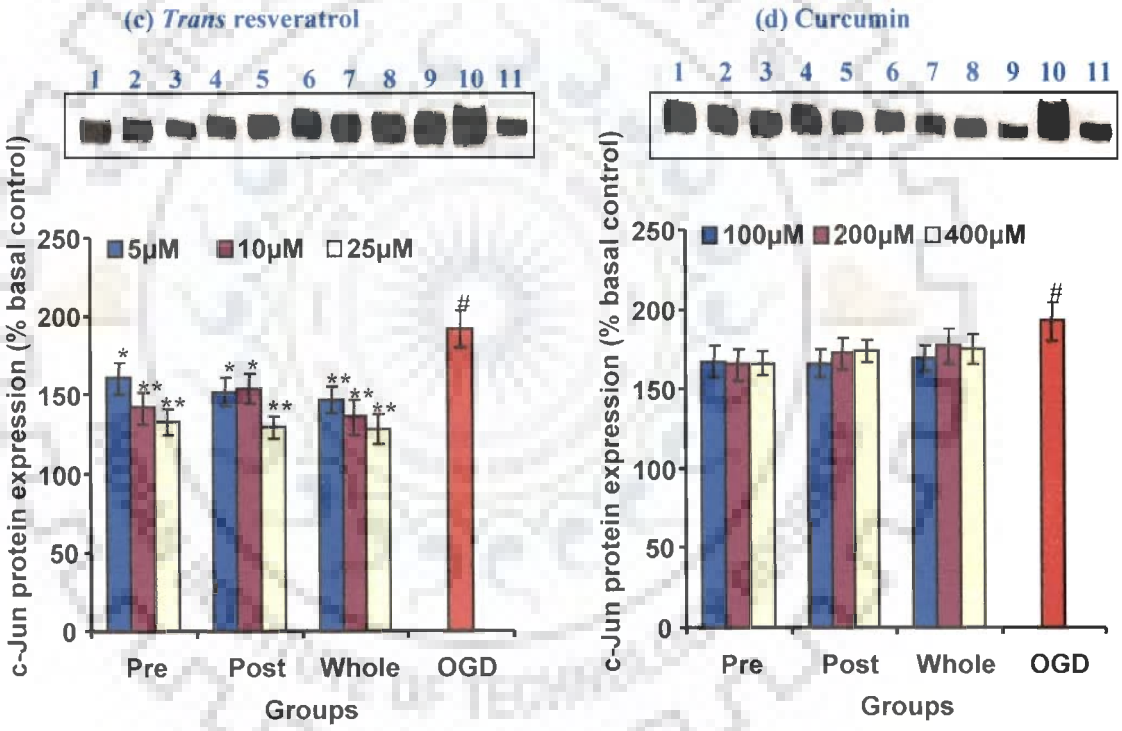


Fig. 4.31c-d: Protein expression for c-Jun using western blot analysis in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (c) and curcumin (d) treatment

Fig.c:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control.Fig.d:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; #$p<0.05$ when OGD compared with basal control. *$p<0.05$, **$p<0.01$ when compared with OGD

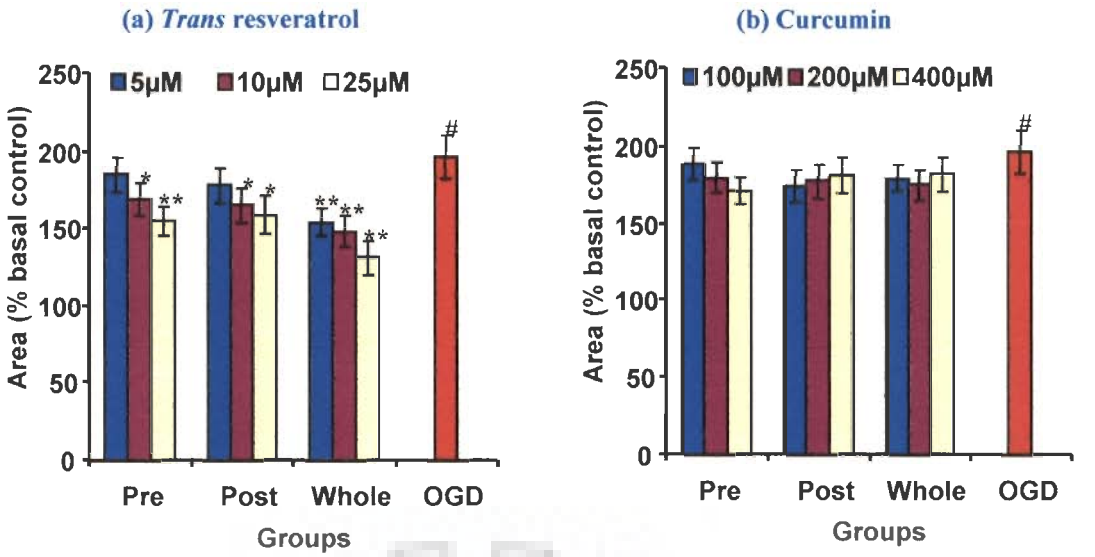


Fig. 4.32a-b: Expression of c-Fos gene protein using immunocytochemical technique in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

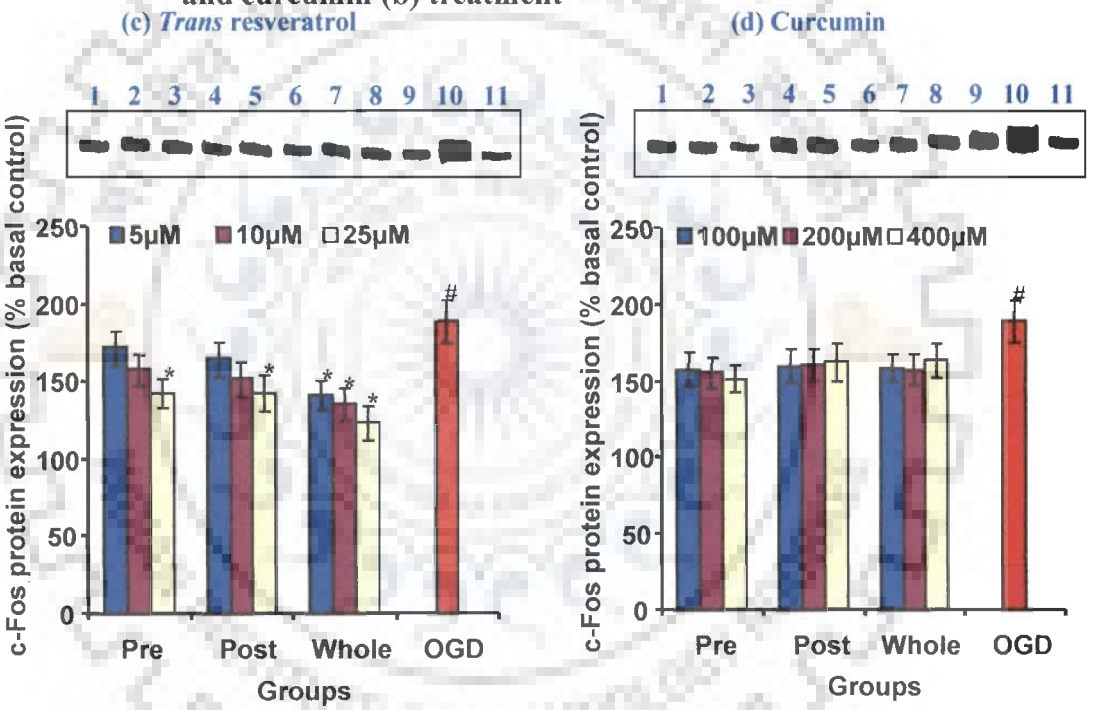


Fig. 4.32c-d: Protein expression for c-Fos using western blot analysis in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (c) and curcumin (d) treatment

Fig.c:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control.Fig.d:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; #$p<0.05$ when OGD compared with basal control. *$p<0.05$, **$p<0.01$ when compared with OGD

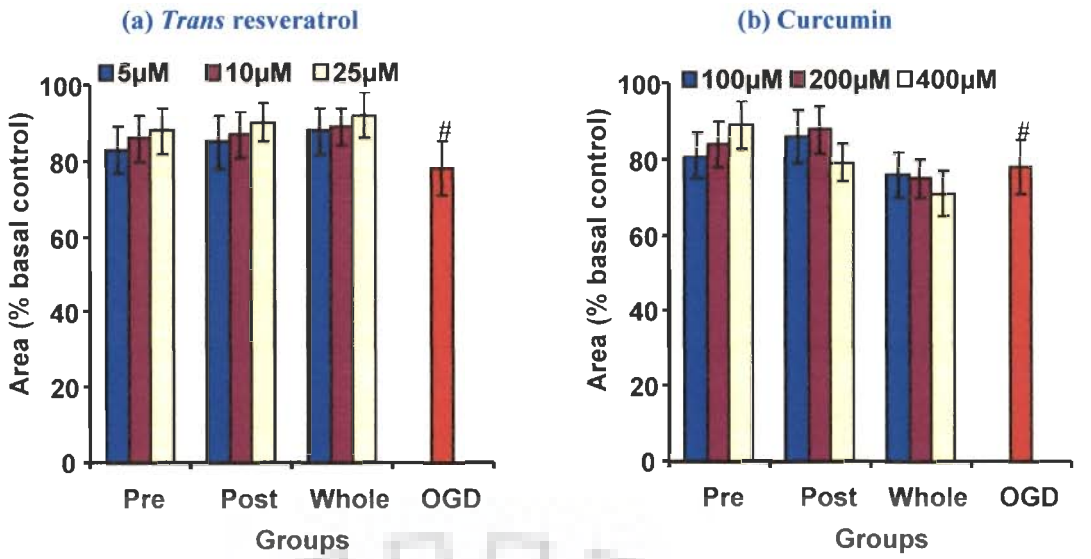


Fig. 4.33a-b: Expression of Bcl-2 gene protein using immunocytochemical technique in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

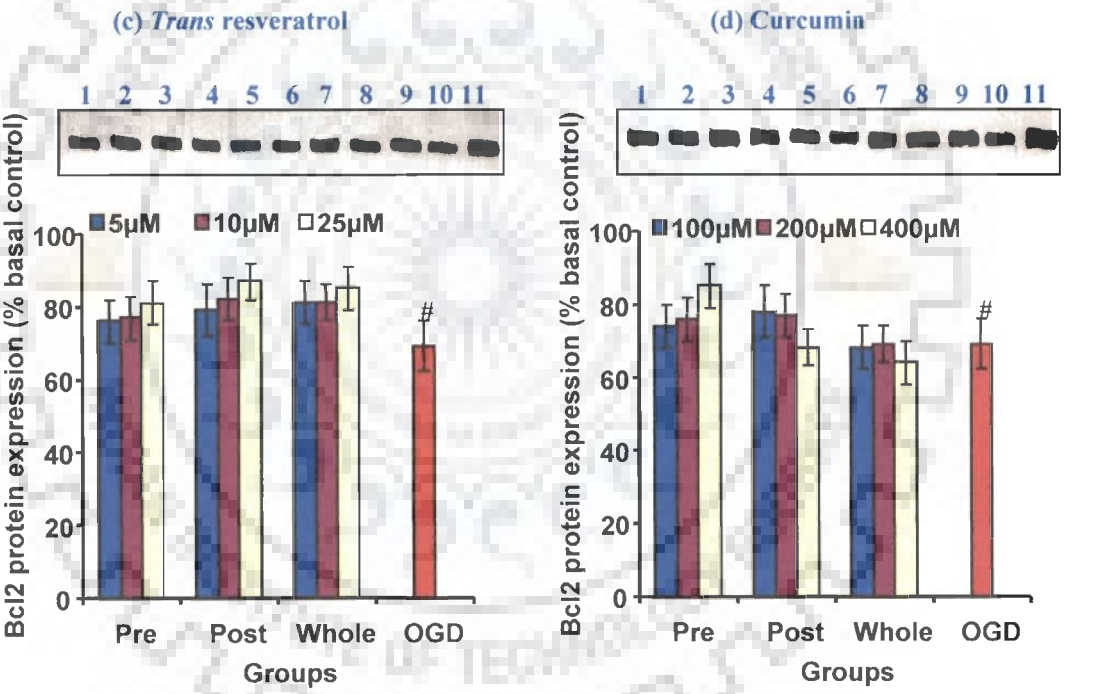


Fig. 4.33c-d: Protein expression for Bcl-2 using western blot analysis in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (c) and curcumin (d) treatment

Fig.c:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control.Fig.d:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; #$p<0.05$ when OGD compared with basal control.

decrease in the expression could be recorded i.e. 71.2±5.4% of normoxia control at 25 μM (Fig. 4.33b).

Western blot analysis for Bcl-2 protein: A significant reduction in the expression of Bcl-2 gene protein could be detected in OGD group (69.6±5.8% of normoxia control). Treatment induced enhancement in the expression was dose independent (Fig. 4.33c). A more or less similar trend following curcumin treatment was recorded for Bcl-2 protein (Fig. 4.33d).

Immunocytochemical quantification of Bax and GAP-43 proteins: OGD induced changes were not so prominent for Bax and GAP-43 proteins in PC12 cells (138.3±12.7% and 122.4±9.6% of normoxia control respectively). None of the treatment schedule at any concentration level for both the drugs used was found capable enough to modulate the changes occurring in the expression of Bax and GAP-43 due to OGD insult in PC12 (Fig. 4.34-4.35a-b).

Western blot analysis for Bax and GAP-43 proteins: Results of western blot analysis for Bax and GAP-43 exhibited similar trends as in case of immunocytochemistry. Insignificant induction in the expression of Bax and GAP-43 gene proteins in OGD group was observed i.e. 129.3±13.6% and 114.7±10.2% of normoxia control respectively. None of the treatment schedule at any dose level for both the drugs used was found capable enough to modulate the changes occurring in the expression of Bax and GAP-43 due to OGD insult in PC12 cells (Fig. 4.34-4.35c-d).

Immunocytochemical quantification of dopamine (DA-D_2) receptor protein: Following live imaging of the cells bound to monoclonal antibody for DA-D_2 receptor and tagged with fluorescent dye, analysis was done for alterations in the expression of receptor protein. As seen with the receptor binding studies, immunocytochemical analysis also showed a significant reduction (51.6±5.8% of normoxia control) in DA-D_2 receptors in cells of OGD group. A concentration dependent increase in the expression could be seen in pre-treatment group of *trans* resveratrol. No significant response of post and whole-treatment groups could be seen, except in case of 25 μM concentration of *trans* resveratrol on post treated group (71.3±6.8% of normoxia control) (Fig. 4.36a). For curcumin, the trend of expressional changes was similar to *trans* resveratrol, however the magnitude of recovery was higher in pre-treatment

group i.e., 81.4±7.6, 88.6±9.1 and 96.4±8.0% of normoxia control following treatment with 100, 200 and 400 μM respectively (Fig. 4.36b).

Western blot analysis for dopamine (DA-D_2) receptor protein: Western blot analysis also showed a significant reduction (47.8±5.6% of normoxia control) in the expression of DA-D_2 receptor proteins in OGD group. A concentration dependent increase in the expression could be seen in pre-treatment group of *trans* resveratrol. No remarkable response of post and whole-treatment groups could be seen, except in case of 25 μM concentration of *trans* resveratrol on post treated group (68.4±7.1% of normoxia control) (Fig. 4.36c). The trends of expressional changes for curcumin treatment were similar to *trans* resveratrol, however the magnitude of recovery was higher in pre-treatment group i.e. 75.7±7.2, 78.6±8.1 and 84.7±9.2% of normoxia control at doses of 100, 200 and 400 μM respectively (Fig. 4.36d).

***In vivo* studies**

Experiments were also conducted using *in vivo* rat middle cerebral artery occlusion (MCAo) model of cerebral stroke to extrapolate the *in vitro* data received from PC12 cells-OGD system. Along with cellular and molecular responses, neurobehavioral parameters involved in cerebral stroke were also studied to see the changes at physiologically functional levels under the influence of biological safe doses of *trans* resveratrol.

Body weight response: It is clearly appeared in Fig. 4.37 that the loss in body weight was significantly higher in vehicle treated group than *trans* resveratrol pre treated group at 24 h of MCAo. A prophylactic pre-treatment of *trans* resveratrol seems to be capable of better in controling the fall in body weight, when compared with post and whole-treatment groups. After 24 h, the recovery was found to be better in post-treatment group, however the overall recovery was greater in pre-treatment group. Sham group of animals was found to recover from the trauma of surgery with seven days of time with the recovery speed at par to the normal animals (Fig. 4.37).

Motor function tests

Tests conducted to assess the motor functions were: neurological scoring, grip strength and spontaneous locomotor activity. The data for motor function activity are presented in Fig. 4.38-4.40ab.

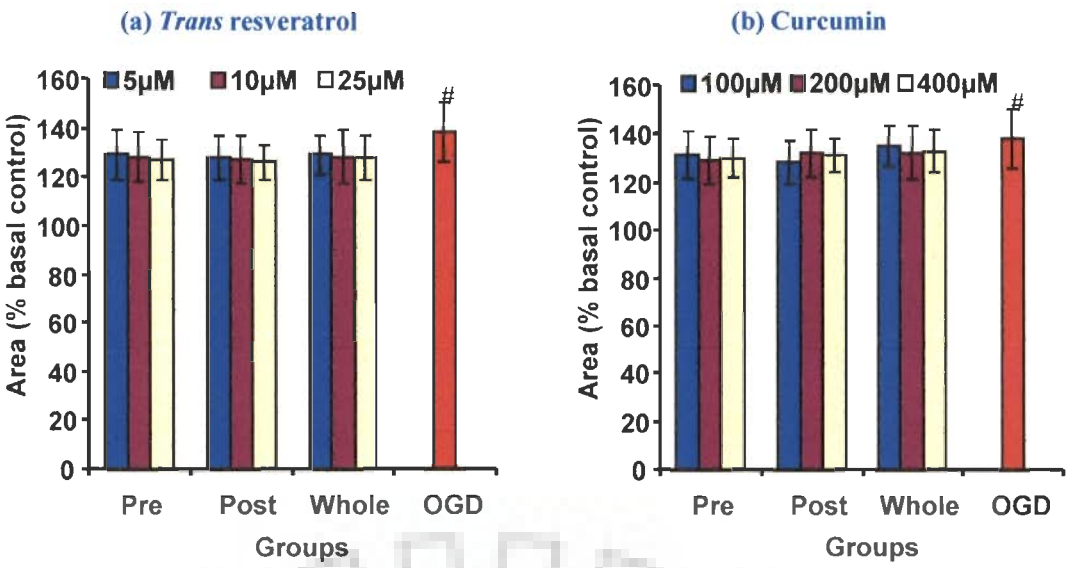


Fig. 4.34a-b: Expression of Bax gene protein using immunocytochemical technique in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

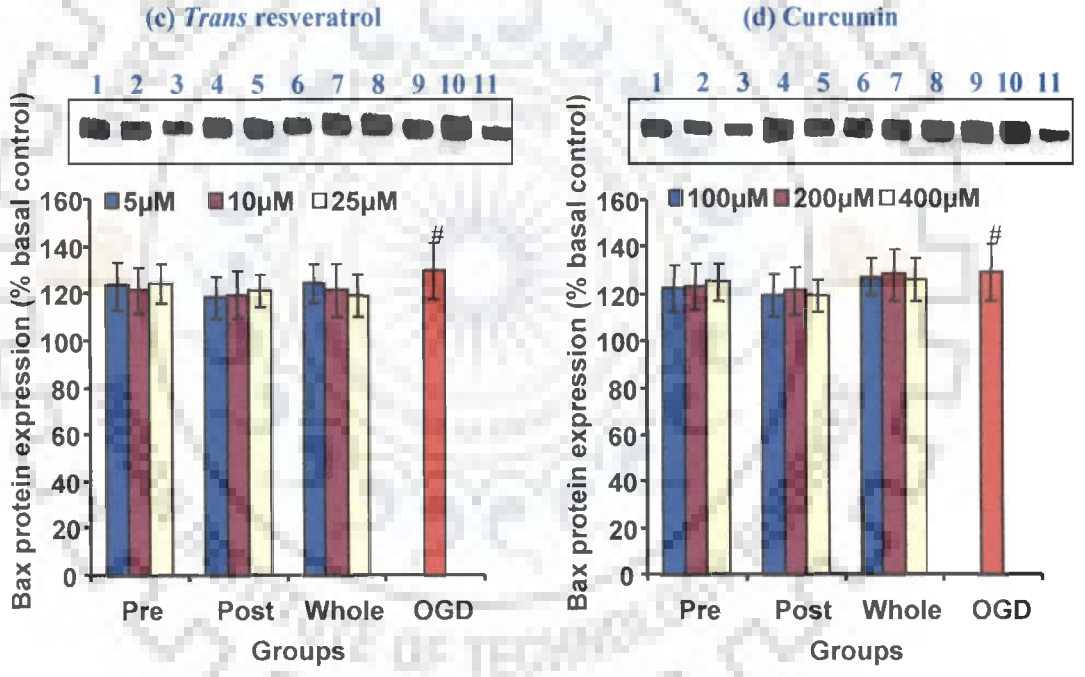


Fig. 4.34c-d: Protein expression for Bax using western blot analysis in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (c) and curcumin (d)treatment

Fig.c:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control.Fig.d:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; #$p<0.05$ when OGD compared with basal control.

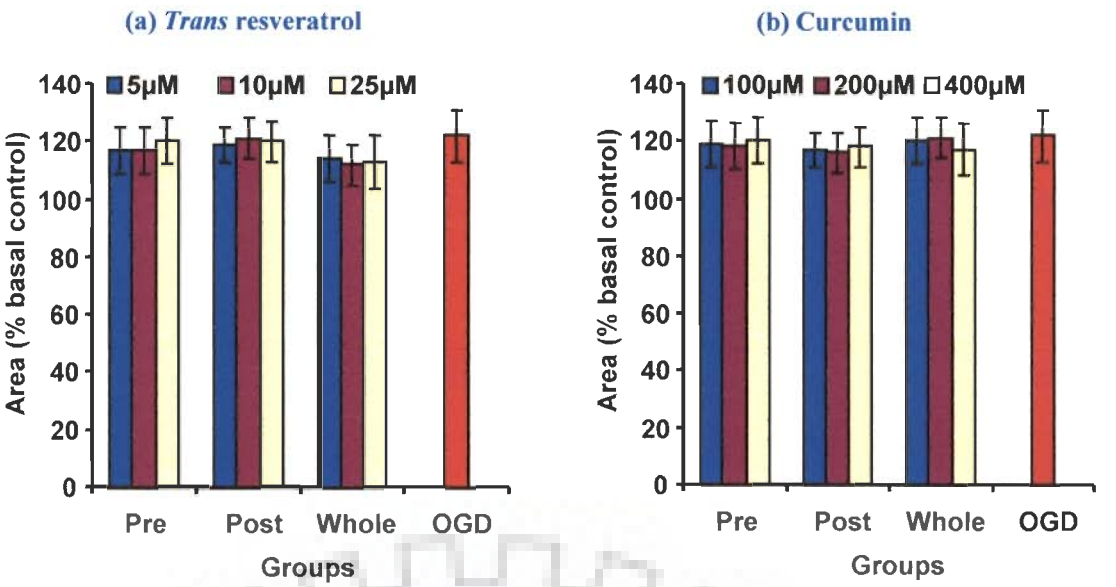


Fig. 4.35a-b: Expression of GAP-43 gene protein using immunocytochemical technique in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b) treatment

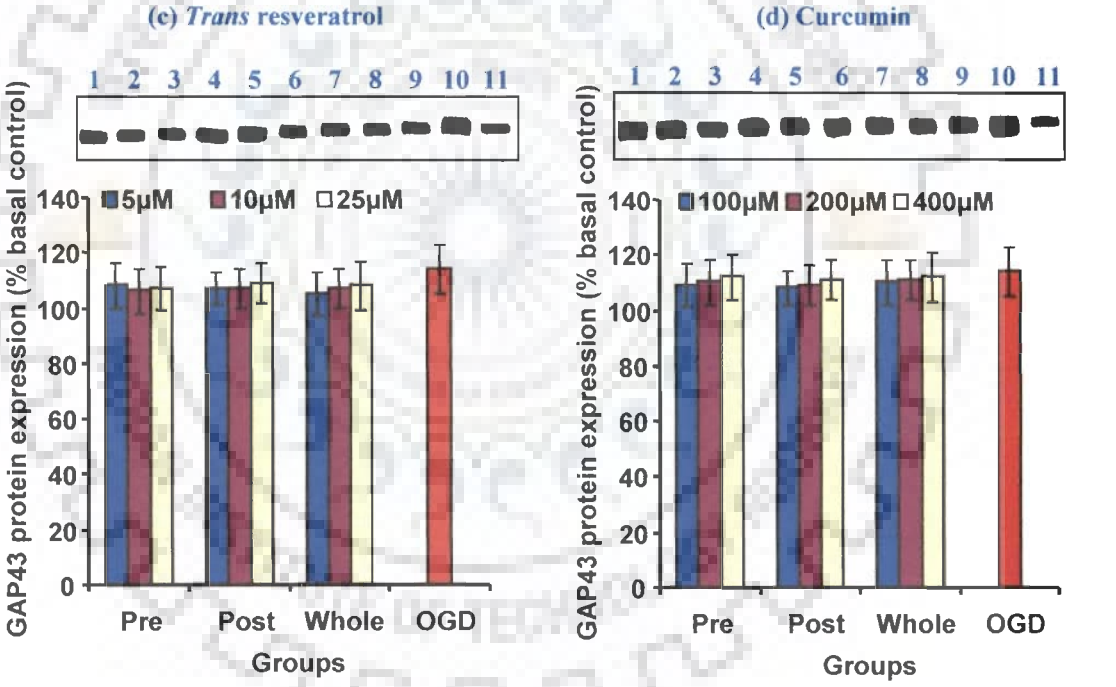


Fig. 4.35c-d: Protein expression for GAP-43 using western blot analysis in PC12 cells following OGD of 6 h and reoxygenation of 24 h and affect of various concentrations of *trans* resveratrol (c) and curcumin (d)

Fig.c:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control. Fig.d:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control.

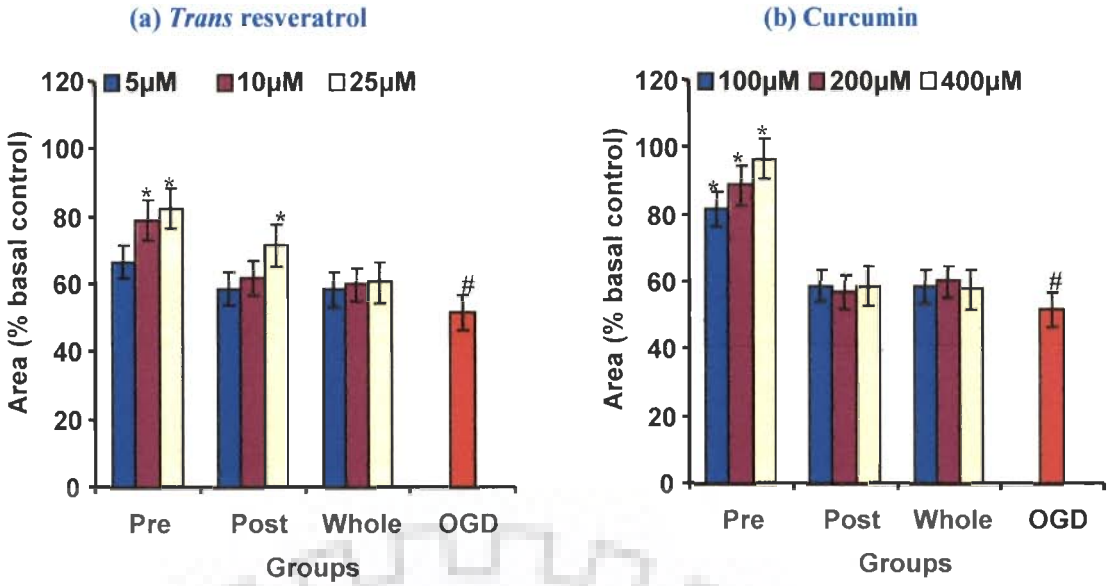


Fig. 4.36a-b: Expression of DA-D_2 gene protein using immunocytochemical technique in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (a) and curcumin (b)treatment

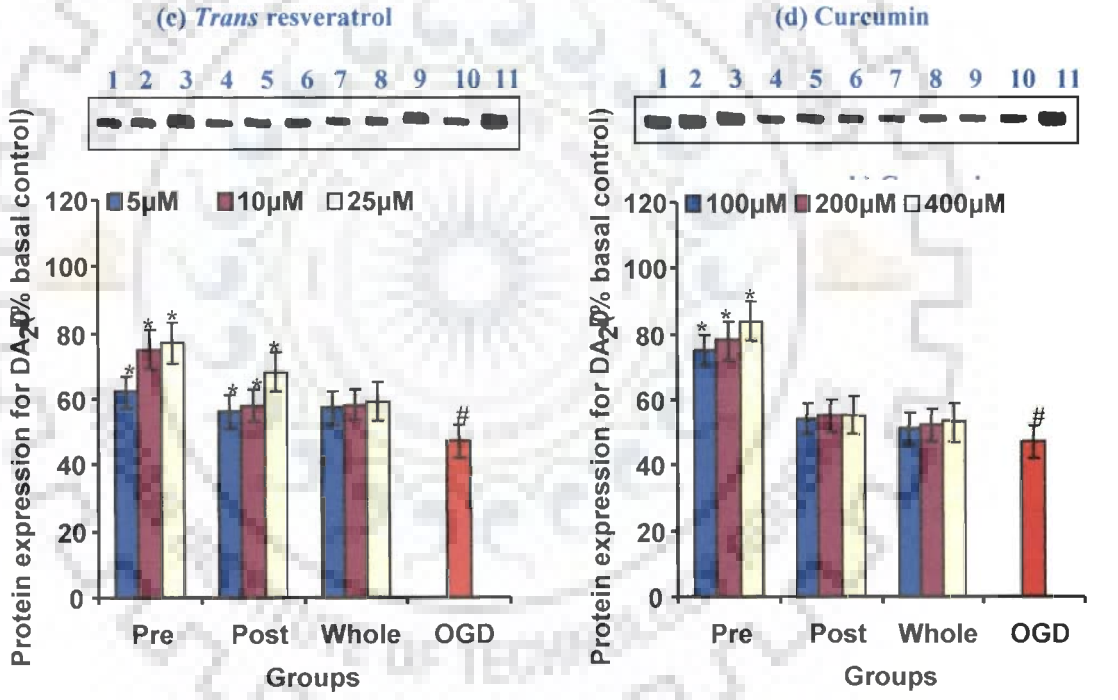


Fig. 4.36c-d: Protein expression for DA-D_2 using western blot analysis in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of various concentrations of *trans* resveratrol (c) and curcumin (d) treatment

Fig.c:Lane 1-3: Pre-treatment of 5, 10 and 25μM; Lane 4-6: Post-treatment of 5, 10 and 25μM; Lane 7-9: Whole-treatment of 5, 10 and 25μM; Lane 10: OGD control; Lane 11: Basal control.Fig.d:Lane 1-3: Pre-treatment of 100, 200 and 400μM; Lane 4-6: Post-treatment of 100, 200 and 400μM; Lane 7-9: Whole-treatment of 100, 200 and 400μM; lane 10: OGD control; Lane 11: Basal control; #$p<0.05$ when OGD compared with basal control. *$p<0.05$, **$p<0.01$ when compared with OGD

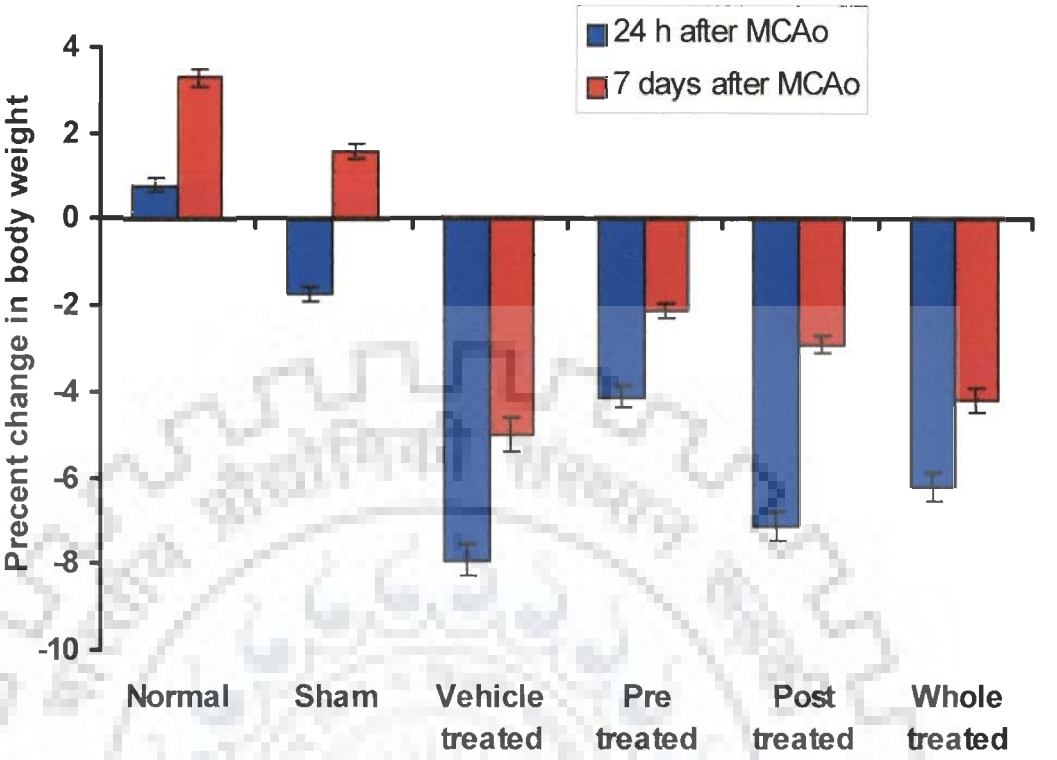


Fig. 4.37: **Effect of *trans* resveratrol on body weight of rats subjected to middle cerebral artery occlusion for 30 min after 24 h and 7 days reperfusion. Values are mean ± SD of seven rats in each group**

Neurological scores: A significant neurological deficit could be recorded in the animals of vehicle treated group (2.2±0.3 neurological scores) when compared with sham control group after 24 h of reperfusion. Among the treatment groups, *trans* resveratrol pre-treatment of 7 days was found to have statistically significant protective effect in experimental rats after 24 h of reperfusion. None of the other treatment schedules could bring down the neurological score up to statistically significant levels (Fig. 4.38a).

With the increase of the reperfusion period i.e. 7 days, a further reduction in neurological scores in pre-treatment group was very prominent, however an auto reversal was also observed in vehicle group. In other treatment groups, a reduction in neurological scores was also recorded but it could not reach to significant levels when compared with vehicle treated group (Fig. 4.38b).

Grip strength: A drastic and highly significant reduction in the grip strength activity was observed in vehicle treated animal when compared with sham operated animals. Like results obtained for neurological scores, in grip strength test also pre-treatment group was found to be most effective, which improves the grip strength from 546±64 lb (vehicle treated) to 712±68 lb (pre treated). However, the improvement in grip strength was significantly lower than the values of sham control (865±79 lb) (Fig. 4.39a).

After 7 days of reperfusion, a reasonably fair increase in grip strength was seen in the animals of pre treated group (821±68 lb) when compared with the sham control animals (881±79 lb). With the passage of time, a mild self recovery in the grip strength was also seen in vehicle treated group (628±64 lb). Increase in the values of post and whole-treatment groups were insignificant (Fig. 4.39b).

Spontaneous locomotor activity: Under the influence MCAo, the spontaneous locomotor activity was brought down to more than 50% (98±12 cm/5 min) in comparison to the sham operated control (212±19 cm/5 min) after 24 h of reperfusion. Following 24 h reperfusion, none of the treatment group was found to have significant protective potential except marginal changes in pre-treatment group. As in case of other activities studied for motor function, a self recovery in vehicle treatment group could be recorded post 7 days of MCAo. A proportional increase in the distance traveled was also observed in pre-treatment group followed by whole-treatment and

post-treatment. In general, efficacy of *trans* resveratrol towards the restoration of distance traveled was not so impressive (Fig. 4.40a-b).

Oxidative stress markers

The involvement of oxidative stress in the causation of neuronal damage during reperfusion is well established and *trans* resveratrol also known to have reducing potential for oxidative stress in variety of neuronal disorders. Thus, attempts were made to assess the suitability of *trans* resveratrol as anti-stroke drug in experimental MCAo model of cerebral stroke. The results for alterations in oxidative stress markers are summarized in Fig. 4.41-4.44a-b.

Malondialdehyde levels: Following 24 h of reperfusion, more than 2 fold increase in the MDA levels were detected in vehicle treated group (498±29 nmol/g wet tissue) in comparison to sham-operated animals (220±21 nmol/g wet tissue). Pre and whole-treatment groups of *trans* resveratrol were found to significantly reduce the MDA levels i.e. 310±26 and 305±24 nmol/g wet tissue, respectively at the end of 24 h of reperfusion. Significant self recovery could be seen in vehicle treated group following 7 days of MCAo. By 7 days of reperfusion period, among the treatment groups maximum reduction in the levels of MDA was observed in pre-treatment group followed by whole and post treated group (252±26, 299±24, 345±23 nmol/g wet tissue, respectively). However, the magnitude of reduction was highest in self-recovery vehicle treated group, thus in general the efficacy of *trans* resveratrol seems to be low or moderate in comparison to other markers studied (Fig. 4.41a-b).

Glutathione levels: Like in case of MDA, only pre-treatment group was found to have potential to restore the glutathione levels near to sham operated group after both 24 h and 7 days of reperfusion (72.7±8.9 and 79.6±8.1 μg/g wet tissue, respectively). Post and whole-treatment groups exhibited a detrimental response by increasing the GSH levels more than vehicle treated group after both 24 h and 7 days of reperfusion (Fig. 4.42a-b).

Superoxide dismutase (SOD) activity: MCAo insult reduced the SOD activity by approximate 50% (21.5±2.6 U/mg protein) in vehicle treated group in comparison to sham operated animals (39.7±3.9 U/g protein). As in case of other endpoints, pre-treatment with *trans* resveratrol was found to be most efficient in the restoration of SOD activity after both 24 h (30.2±2.3 U/g protein) and 7 days (32.3±2.6 U/g protein)

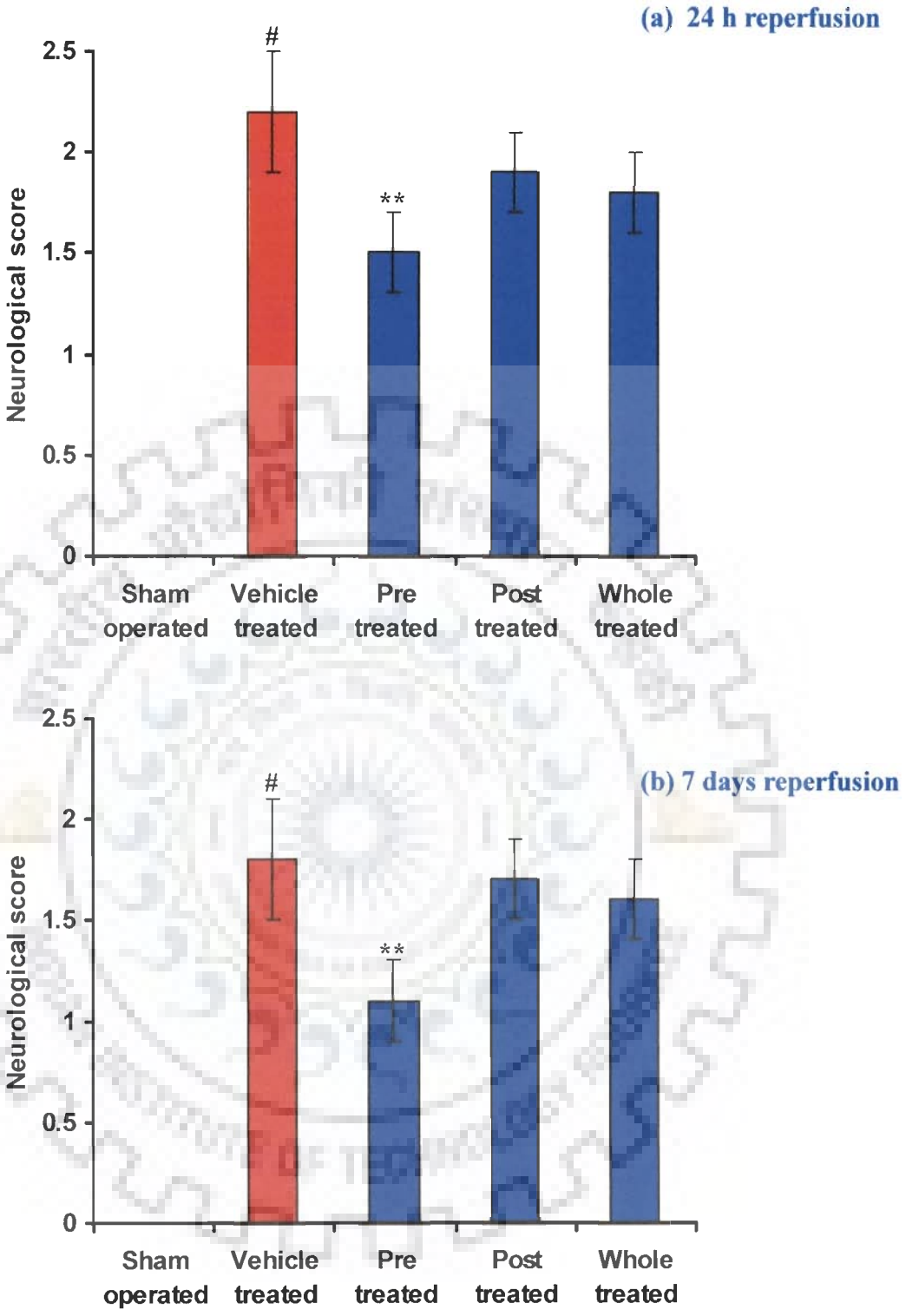


Fig. 4.38: **Effect of *trans* resveratrol on neurological scores in rats subjected to middle cerebral artery occlusion for 30 min and 24 h (a) and 7 days (b) after ischemic reperfusion injury. Values are mean ± SD of seven rats in each group**

#p<0.05 when vehicle treated group compared with sham operated group
**p<0.01 when compared with vehicle treated rats

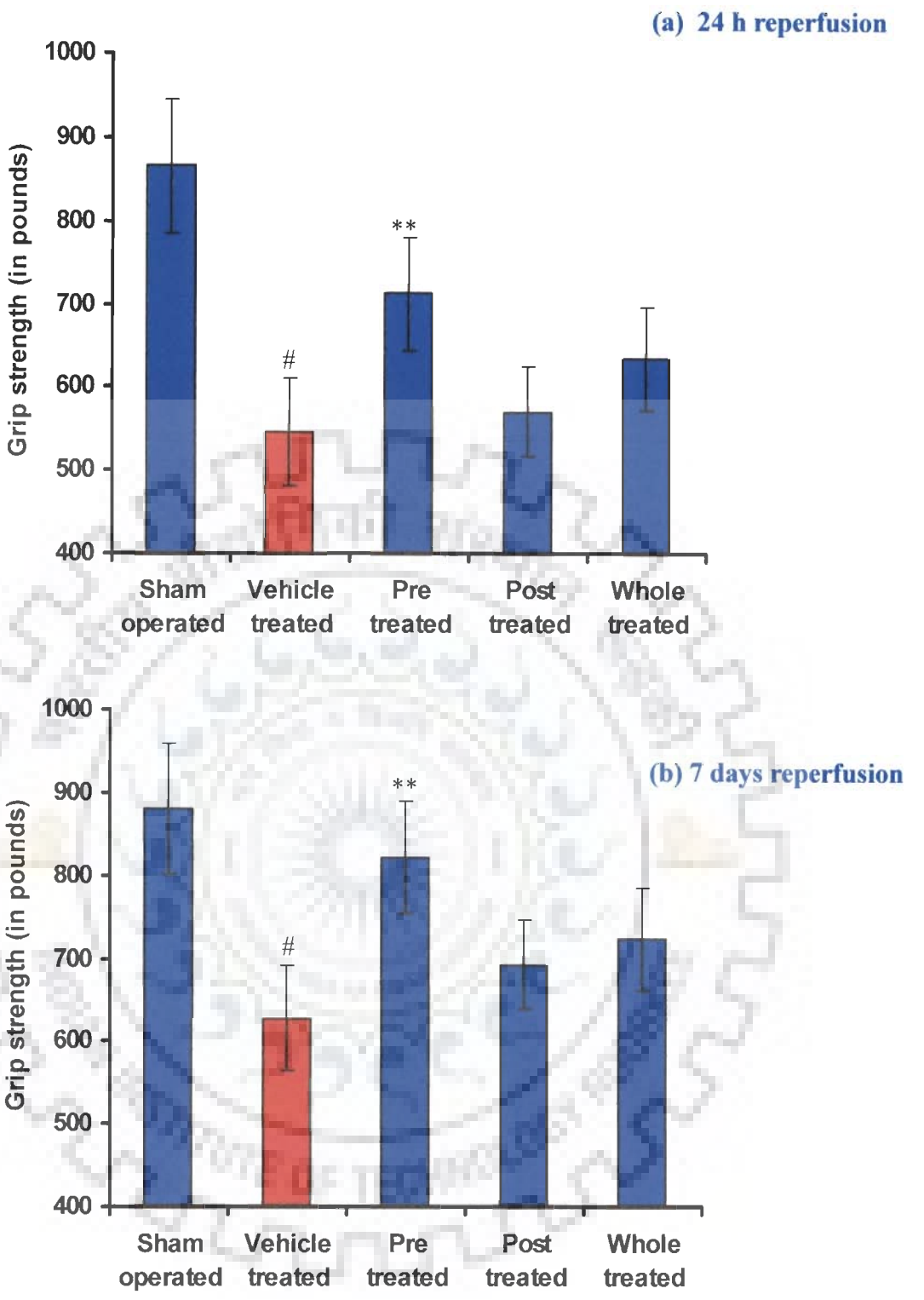


Fig. 4.39: **Effect of *trans* resveratrol on grip strength in rats subjected to middle cerebral artery occlusion for 30 min and 24 h (a) and 7 days (b) after ischemic reperfusion injury. Values are mean ± SD of seven rats in each group**

#p<0.05 when vehicle treated group compared with sham operated group

**p<0.01 when compared with vehicle treated rats

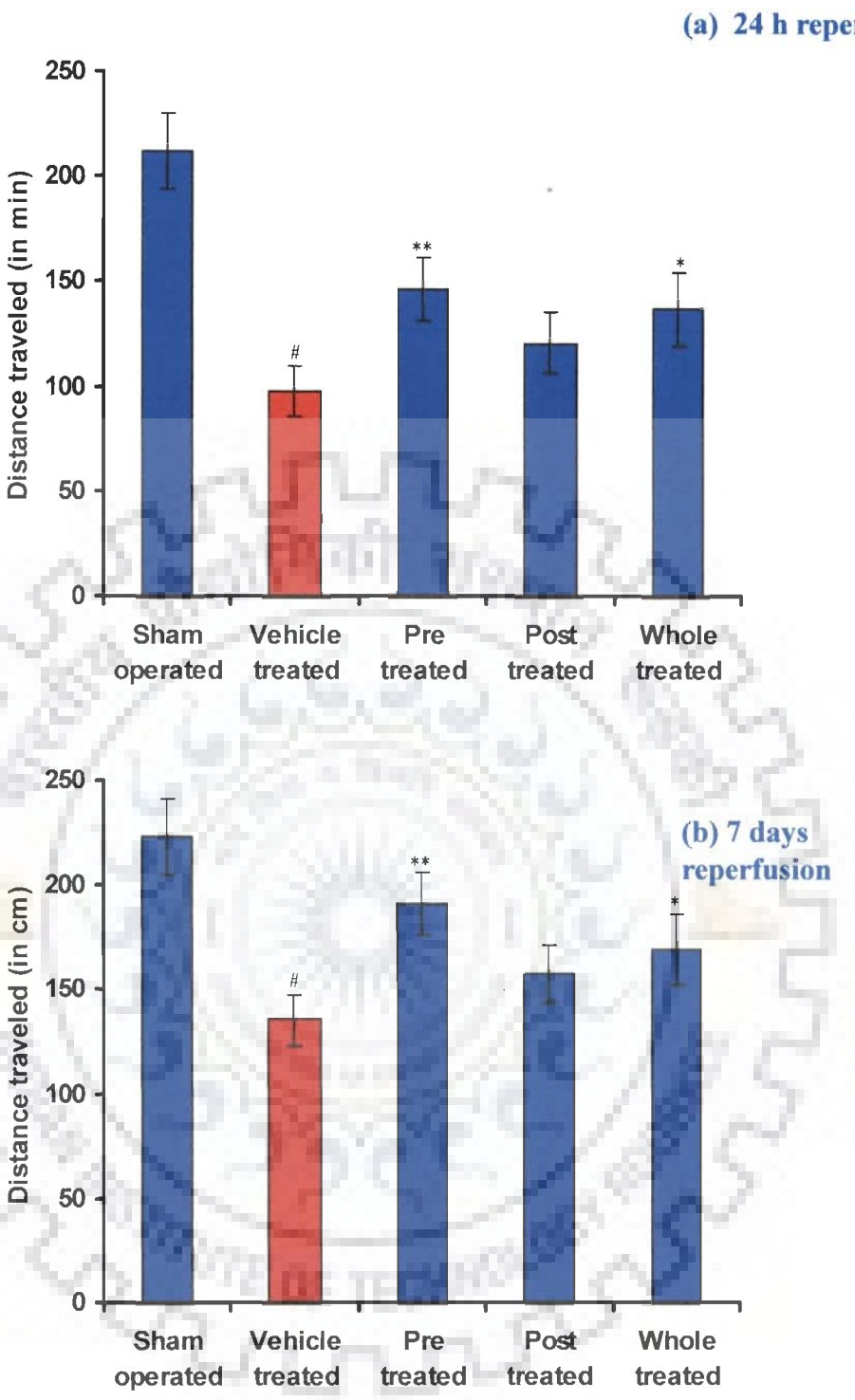


Fig. 4.40: **Effect of *trans* resveratrol on locomotor activity in rats subjected to middle cerebral artery occlusion for 30 min and 24 h (a) and 7 days (b) after ischemic reperfusion injury. Values are mean ± SD of seven rats ineach group.**

#p<0.05 when vehicle treated group compared with sham operated group

*p<0.05, **p<0.01 when compared with vehicle treated rats

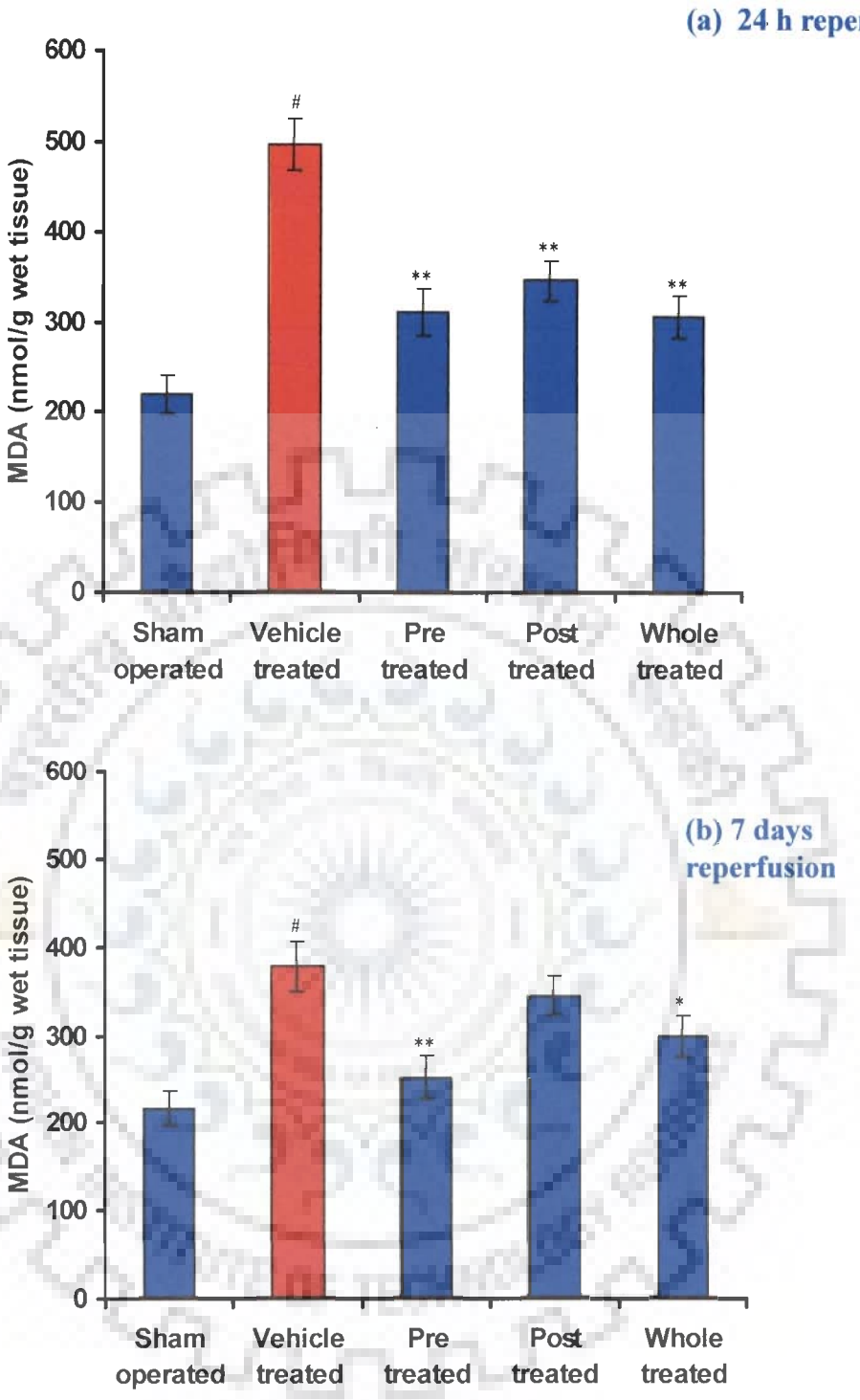


Fig. 4.41: **Effect of *trans* resveratrol on melondialdehyde levels in rats subjected tomiddle cerebral artery occlusion for 30 min and 24 h (a) and 7 days (b) after ischemic reperfusion injury. Values are mean ± SD of seven rats in each group.**

#p<0.05 when vehicle treated group compared with sham operated group
*p<0.05, **p<0.01 when compared with vehicle treated rats

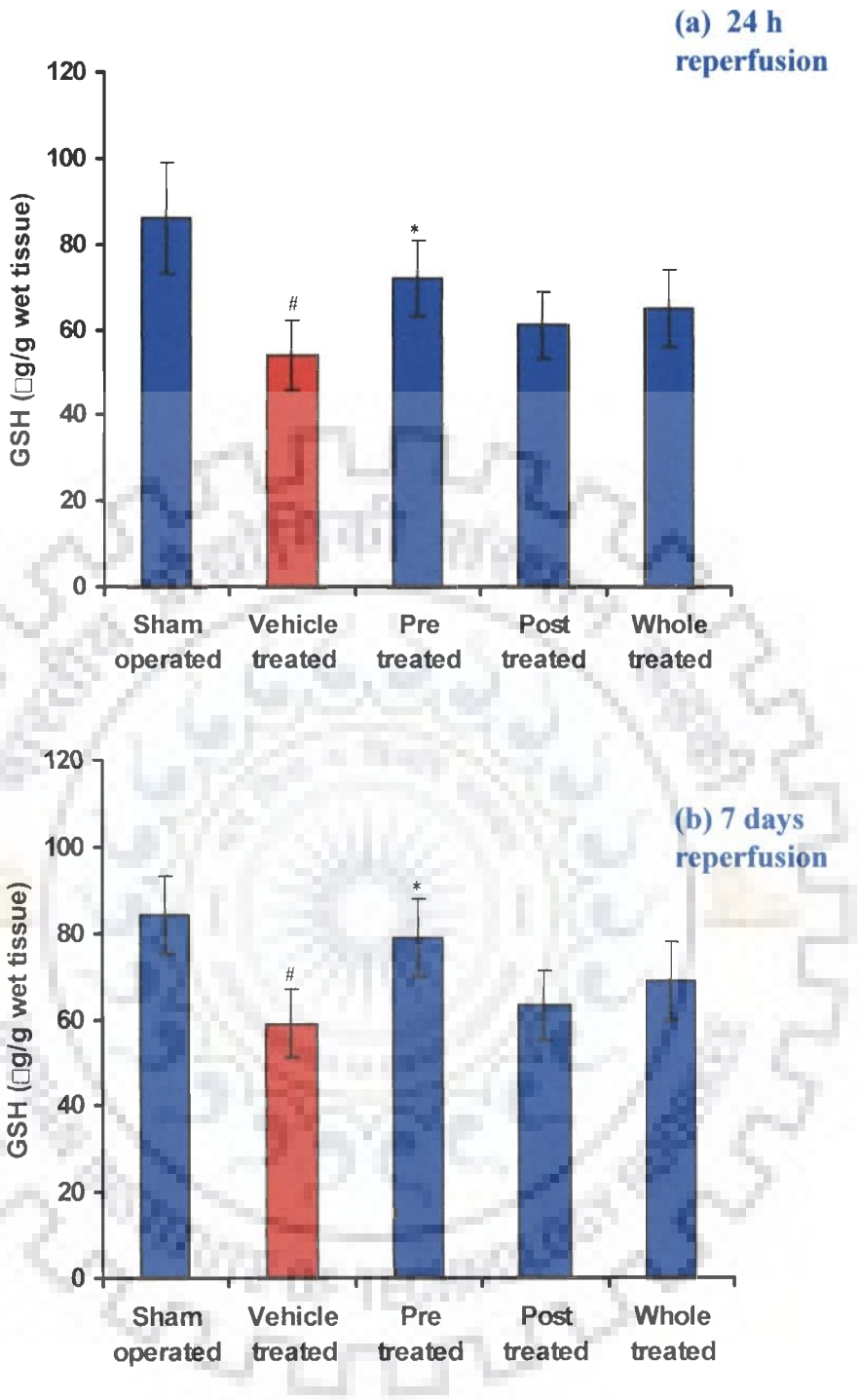


Fig. 4.42: **Effect of *trans* resveratrol on glutathione levels in rats subjected to middle cerebral artery occlusion for 30 min and 24 h (a) and 7 days (b) after ischemic reperfusion injury. Values are mean ± SD of seven rats in each group.**

#p<0.05 when vehicle treated group compared with sham operated group

*p<0.05 when compared with vehicle treated rats

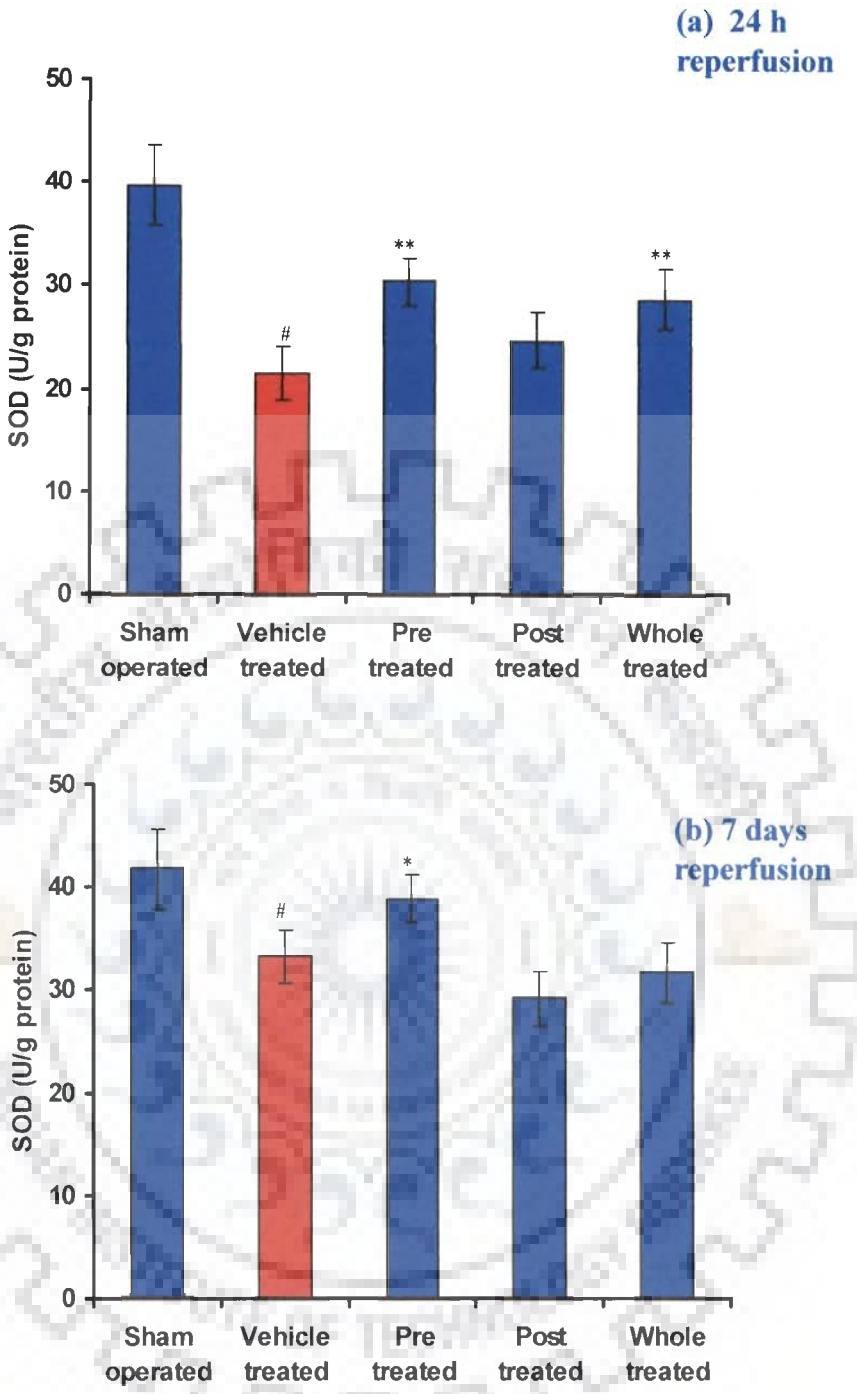


Fig. 4.43: **Effect of *trans* resveratrol on superoxide dismutase activity in rats subjected to middle cerebral artery occlusion for 30 min and 24 h (a) and 7 days (b) after ischemic reperfusion injury. Values are mean ± SD of seven rats in each group.**

#p<0.05 when vehicle treated group compared with sham operated group

*p<0.05, **p<0.01 when compared with vehicle treated rats

of reperfusion. The post and whole-treatment groups could not elicit any significant changes. Self recovery in vehicle treated group was not observed with the extended reperfusion period of 7 days (Fig. 4.43a-b).

Catalase activity: A significant recovery in the catalase activity was reported in pre-treatment group (35.9±3.7 U/g protein) when compared with vehicle treated groups (21.5±2.6 U/g protein) after 24 h of reperfusion period. For catalase activity, whole- and post-treatment groups were also reasonably good after 24 h of reperfusion (28.7±2.9, 24.8±2.7 U/g protein, respectively). Although, the magnitude of recovery was greater in pre-treatment group, the magnitude of self recovery in vehicle treated group was greater than pre-treatment group following 7 days of MCAo (Fig. 4.44a-b).

^{3}H-spiperone binding (DA-D_2 receptor levels): The binding of ^{3}H-spiperone, specific radioligand for DA-D_2 receptors in normal rats (without any surgery) was found to be 429±33 pmol/g protein. The value for dopamine receptor binding in sham operated rats was 446±29 pmol/g protein (105.9±9.3% of normal control). The response of *trans* resveratrol treatment on dopamine receptor binding was calculated by comparing the values with normal control and expressed in percentage.

Following 24 h of MCAo, the levels of DA-D_2 receptors in vehicle treated group was reduced to 56.5±5.8% of control. Among the treatment groups, pre-treatment of *trans* resveratrol resulted in elevation of DA-D_2 receptor values up to 88.2±7.6% of normal control; whereas in post and whole-treatment groups, the values were little lower than pre-treatment i.e. 66.0±5.9 and 73.5±6.6% respectively of control.

After 7 days of reperfusion, a generalized self and *trans* resveratrol induced restoration in the values of DA-D_2 receptor could be recorded, except in case of vehicle treatment group where a mild decrease in the DA-D_2 receptor level was observed (51.2±4.9% of control). Animals pre-treated with *trans* resveratrol for 7 days exhibited receptor binding up to 91.3±7.9% of control at day 7 of reperfusion. A moderate increase in the receptor binding was also observed in post (79.2±6.3% of control) and whole-treatment (83.3±7.1% of control) groups by day 7 of reperfusion. In general, *trans* resveratrol treatment for 7 days prior to MCAo was found to be most effective among all the treatment groups (Fig. 4.45a-b).

Infarction volume analysis in brain slices

Histological examination of brain slices by TTC staining was done for the analysis of MCAo induced infarction volume and restorative potential of *trans* resveratrol by 24 h and 7 days of reperfusion. A significant infarction volume (152.4±23.3 mm^3) was seen in vehicle treated group following 24 h of MCAo. A highly significant decrease in the infarction volume could be recorded in pre-treatment group (64.6±14.2 mm^3) followed by whole (69.5±12.1 mm^3) and post-treatment (83.7±16.2 mm^3) groups respectively (Fig. 4.46a).

After 7 days of reperfusion, animals received vehicle treatment exhibited a tremendous increase in the infarction volume (173.8±27.3 mm^3). On the other hand, pre-treatment group was found to able to control the increase of infarction volume i.e. 79.3±18.2 mm^3 (an insignificant increase than the values of same group at 24 h reperfusion). The efficacy response of post and whole-treatment groups was also similar as in case of 24 h of reperfusion (Fig. 4.46b).

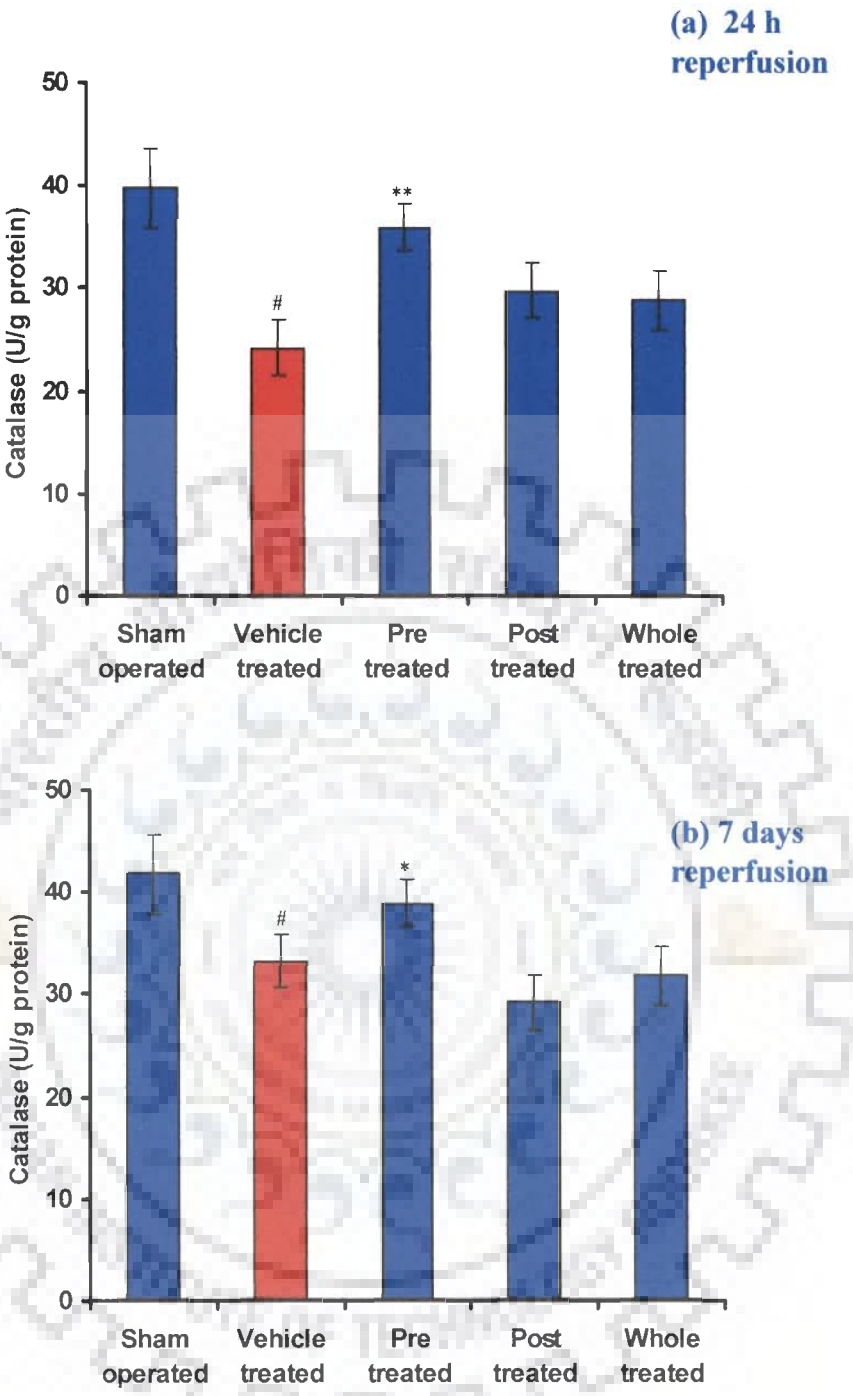


Fig. 4.44: **Effect of *trans* resveratrol on catalase activity in rats subjected to middle cerebral artery occlusion for 30 min and 24 h (a) and 7 days (b) after ischemic reperfusion injury. Values are mean ± SD of seven rats in each group**

#p<0.05 when vehicle treated group compared with sham operated group

*p<0.05, **p<0.01 when compared with vehicle treated rats

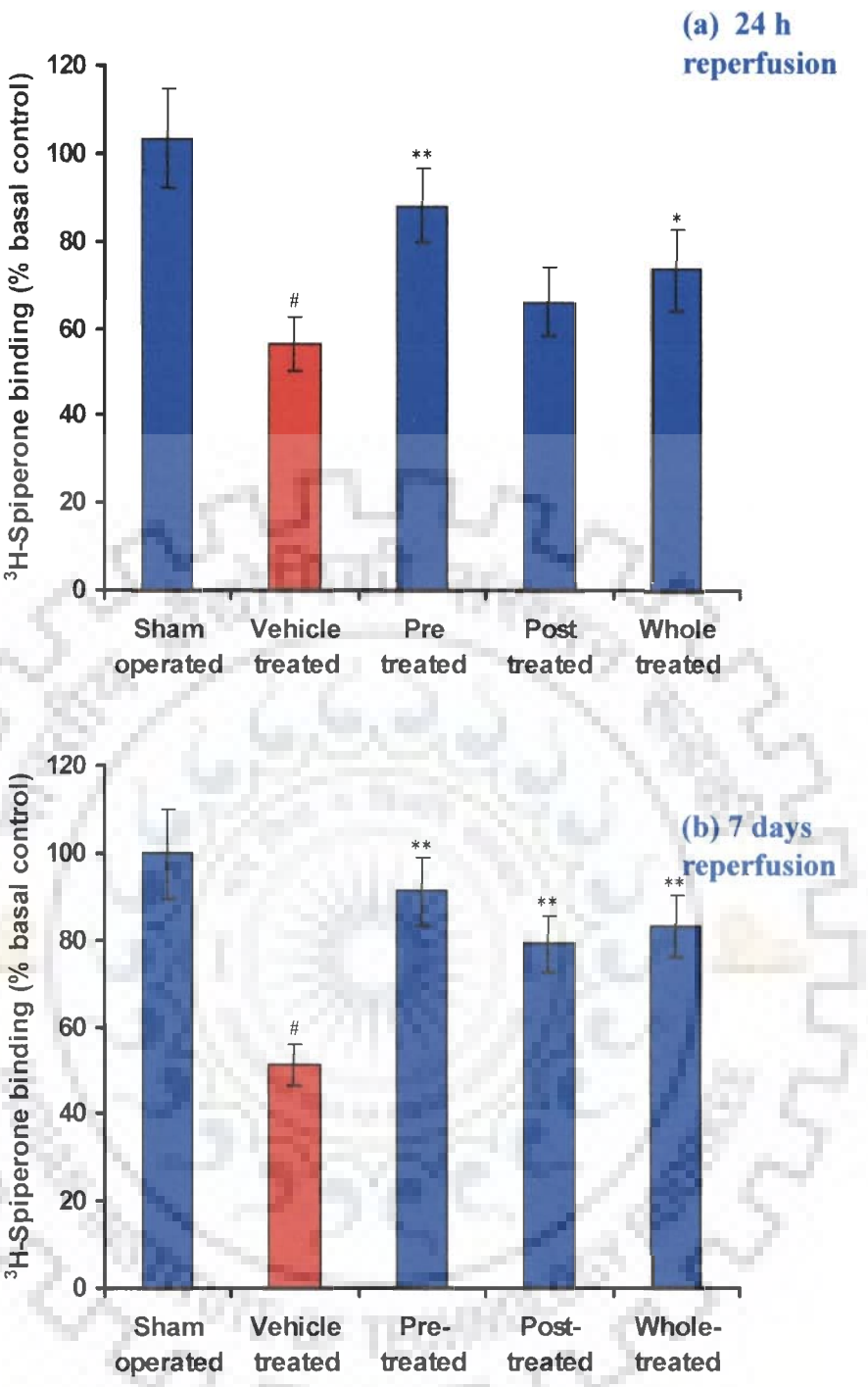


Fig. 4.45: **Effect of *trans* resveratrol on DA-D_2 receptor levels in rats subjected to middle cerebral artery occlusion for 30 min and 24 h (a) and 7 days (b) after ischemic reperfusion injury. Values are mean ± SD of seven rats in each group.**

#p<0.05 when vehicle treated group compared with sham operated group

*p<0.05, **p<0.01 when compared with vehicle treated rats

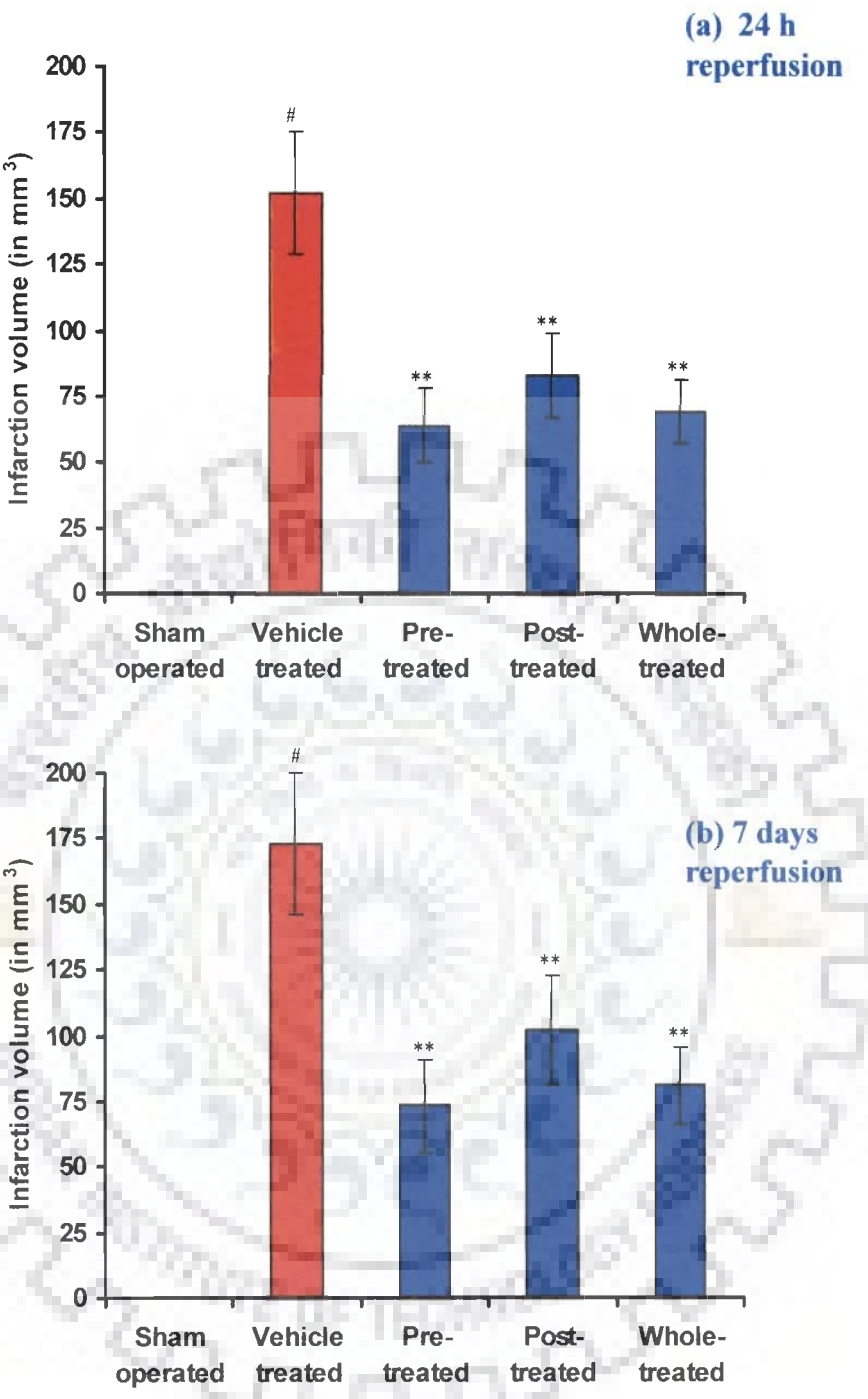


Fig. 4.46: **Effect of *trans* resveratrol on infarction volume in rats subjected to middle cerebral artery occlusion for 30 min and 24 h (a) and 7 days (b) after ischemic reperfusion injury. Values are mean ± SD of seven rats in each group.**

#p<0.05 when vehicle treated group compared with sham operated group

**p<0.01 when compared with vehicle treated rats

Discussion

5. DISCUSSION

Optimization of oxygen glucose deprivation (OGD) conditions in PC12 cells

Effect of OGD on cell viability

Results of trypan blue dye exclusion test are suggestive that the loss of cell viability in cultured PC12 cells was initiated from an OGD of 2 h and gradually reached statistically significant levels by 6 h, with the increase in OGD period (7 and 8 h) this effect became more intense. Thus, an OGD period of 6 h was selected for further studies. Tabakman *et al.* (2002) have shown 48% loss of cell viability in PC12 cells following 4 h OGD insult under anoxia conditions whereas present study could elicit a similar effect by 6 h OGD under hypoxia (1% O_2). The severity of anoxia over hypoxia may be one of the reasons for this difference in findings, even with the same cells, i.e. PC12 cells. Besides this, many other factors may also be responsible for such differences including the metabolic state of cells, passage number and availability of intracellular antioxidants or external support of these antioxidants from culture medium (Koubi *et al.*, 2005; Shi *et al.*, 2006; Tagliari *et al.*, 2006; Tjong *et al.*, 2007). Cells of different origin and types have also been employed to study the difference, if any, in response to OGD insult (Ito *et al.*, 2006; Kang *et al.*, 2006). Several workers have used 3 h OGD period to study the loss of integrity in primary cultures of rat brain neuronal cells (Briyal *et al.*, 2006; Luo *et al.*, 2006). An immortalized cell line, HN33, derived from somatic cell fusion of mouse hippocampal neurons and N18TG2 neuroblastoma cells have shown ~20% viable cells even after an OGD of 24 h (Jin *et al.*, 2000). The primary co-cultures of human cortical neuronal and glial cells were found to have similar responses for cell viability like NH33 cell line within a period of 50 min of OGD insult (Shi *et al.*, 1998). Similarly, in brain hippocampal slices of rats, a significant reduction in cell viability could be recorded with 45 min OGD followed by 3 h reoxygenation (Werth *et al.*, 1998; Tagliari *et al.*, 2006). Thus, cell-specific responsiveness to the OGD insult can be suggested.

Influence of glucose concentrations during reoxygenation period

Glucose concentrations between 4-6 mg/ml in culture medium were found to augment maximum cell growth during reoxygenation period. Following the 6 h OGD insult, cells growing in culture medium having no glucose seem to be starved even after 6 h of reoxygenation and decrease in cell viability continued till the end of incubation period, i.e. 96 h. It is well documented that in ischemic cerebral stroke,

during the reoxygenation period, glucose metabolism is very high in the brain, especially in early hours, to perform the phosphorylation of ADP to ATP (Schurr, 2002; Iijima *et al.,* 2003). A large amount of ATPs are required and utilized in the maintenance of intracellular homeostasis (Plaschke *et al.,* 1998; Schurr, 2002), transmembrane ion gradients of sodium, potassium and calcium (Schurr, 2002; Iijima *et al.,* 2003) and other wear and tears like oxidative stress induced damages (Schurr, 2002) in the cells. Under *in vitro* conditions, cells were exposed to glucose directly through culture medium along with oxygen that is supposed to make the system more efficient for rapid recovery and proliferation by performing new glycolysis (Morais *et al.,* 2005; Gao *et al.,* 2007). The availability of oxygen results in speedy conversion of accumulated lactic acid into pyruvic acid in the cytoplasm (Schurr *et al.,* 1997; Fukuchi *et al.,* 1998), which enters into mitochondria and produces ATPs through respiratory chain. At each time point of reoxygenation period, there was a significant metabolic recovery in the batches having glucose concentration between 4-6 mg/ml, however, intra-group variation in the recovery was insignificant at all the time points of the reoxygenation period. This might be due to saturation of enzymes molecules involved in the metabolism of glucose. Further, at every time point of reoxygenation, an additional number of cells were available for glucose metabolism, so there was an overall increase in the recovery, which continued till the end of reoxygenation period for glucose concentration up to 6 mg/ml. Glucose concentrations of 7 mg/ml and above were found to be detrimental in a concentration and time-dependent manner during the reoxygenation period. This observation is in accordance with earlier findings where other workers have demonstrated hyperglycemia induced statistically significant reduction in cell viability in rat brain slices and cultured cells (Pellerin and Magistretti, 1994; Schurr *et al.,* 1997). Similar findings have also been reported with *in vivo* studies using a MCAo rat model of cerebral stroke (Li *et al.,* 1998; Liu *et al.,* 2007). Organopathy, including neuropathy, resulting from hyperglycemia in uncontrolled patients of diabetes mellitus may be a basis to justify this finding in the experimentation (Said, 2007).

Time period and glucose concentrations during reoxygenation period

Following 6 h of OGD, when cells received an exposure to oxygen and glucose, a cascade of events took place including free radical production (Lelkes *et al.,* 2001; Muranyi and Li, 2006), sudden start and aggravation of mitochondrial

respiratory chains (Iijima *et al.,* 2006), excitotoxicity (Fujimoto *et al.,* 2004; Montero *et al.,* 2007), disruption of sodium and calcium influx (Sanchez *et al.,* 2004; Blomgren and Hagberg, 2006), enzymatic changes (Muranyi and Li, 2006), stimulation of the inflammatory process (Gendrona *et al.,* 2004; Chechneva *et al.,* 2006), endothelin release (Wu *et al.,* 2006), endothelial dysfunction (Keep *et al.,* 2005; Chavez *et al.,* 2006) and inhibition of protein synthesis (Beck *et al.,* 2003). The results of the initial few hours of reoxygenation do not convey a real picture of cellular recovery since glucose provides energy for growth and proliferation while oxygen induces free radical production and consequent cellular damage (Blomgren and Hagberg 2006). With the progress of reoxygenation period, recovery started to maintain homeostasis in the effected cells (Chechneva *et al.,* 2006; Zhu *et al.,* 2007). Thus, 24 h of reoxygenation was considered as a true recovery point, since increase in cell viability became statistically significant from this point onwards only and gradually continued till the end of the reoxygenation period, i.e. 96 h. Recent studies conducted using a variety of cell systems have also re-affirmed the suitability of the 18-24 h reoxygenation period (Shi *et al.,* 2006; Fordel *et al.,* 2007; Montero *et al.,* 2007; Zhu *et al.,* 2007).

The data suggest that glucose concentration during reoxygenation may be one among the key factors involved in the growth and proliferation in PC12 cells. The OGD of 6 h followed by a reoxygenation period of 24 h with 4-6 mg/ml glucose concentrations were found to be the optimum conditions to create the experimental conditions similar to cerebral stroke under *in vitro* environment using PC12 cells.

Development of *in vitro* model of cerebral stroke

The pathophysiological changes postulated to occur as a response to stroke are free radical production, excitotoxicity, disruption of sodium and calcium influx, enzymatic changes, stimulation of the inflammatory process, endothelin release, activation of platelets and leukocytes, delayed coagulation and endothelial dysfunction. All of these pathophysiological reactions may contribute to brain injury following the onset of stroke (Scott and Gray, 2000). In order to suggest the usefulness of PC12 cells-OGD system as rapid and sensitive *in vitro* model of cerebral stroke, a number of endpoints involved in the process of causation and progression of cerebral stroke have been studied. Studies confirmed that 6 h of OGD followed by 24 h of reoxygenation period leads to statistically significant cytotoxic responses in PC12 cells. These endpoint or specific responses in variety of *in vitro* systems have been

analyzed as model for ischemic cerebral stroke as reported by various workers viz., primary cultures of rat brain neuronal cells (Briyal *et al.,* 2007), immortal human neuroblasts cell lines like Neuro-2a, SH-SY5Y, (Miglio *et al.,* 2004; Chang *et al.,* 2008) and U-87 (Santra *et al.,* 2006), rat brain slices (Jung *et al.,* 2008; Konrath *et al.,* 2008; Zhang *et al.,* 2008). The findings of the present study are in confirmation with these studies and suggest that many factors may influence the magnitude of OGD including metabolic state of cells, passage number and availability of intracellular antioxidants or external support of these antioxidants from culture medium (Koubi *et al.,* 2005; Shi *et al.,* 2006; Tagliari *et al.,* 2006; Tjong *et al.,* 2007; Wang *et al.,* 2008). Besides, the available percentage of oxygen during OGD is also one of the contributing factors in cytotoxic response. Tabakman *et al.,* (2002) have shown 48% loss of cell viability in PC12 cells following 4 h OGD insult under anoxia conditions whereas one could get a similar effect by 6 h OGD under hypoxia (1% O_2). The severity of anoxia over hypoxia may be one of the reasons for this difference in findings, even with the same cells, i.e. PC12 cells.

The pathophysiology of ischemic cerebral stroke has also been associated with ROS generation mediated oxidative damage (Doyle *et al.,* 2008). Thus, in the present study, markers related to oxidative stress were assessed following 6 h of OGD and 24 h of reoxygenation. It has been observed that PC12 cells, under experimental conditions, respond by increasing ROS and NO production up to 230.6±13.4% and 133.2±8.1% of normoxia control respectively, whereas significant decrease could be observed in catalase, superoxide dismutase and glutathione levels when compared to normoxia control. These findings are indicative of oxidative stress mediated cytotoxicity in cells. Production of reactive oxygen species is known to increase, sometimes drastically, leading to tissue damage via several different cellular and molecular pathways (Doyle *et al.,* 2008). The damage could have become more widespread due to weakened cellular antioxidant defense systems after ischemic stroke (Schaller, 2005).

After brain injury by ischemic stroke, the production of free radical increases through several different cellular pathways, including calcium activation of phospholipases, nitric oxide synthase, xanthine oxidase and the Fenton and Haber-Weiss reactions by inflammatory cells, leading to tissue damage (Doyle *et al.,* 2008). Oxidative damage does not occur in isolation but participates in the complex interplay

between excitotoxicity, apoptosis and inflammation during ischemia and reperfusion (Barone and Feuerstein, 1999; Doyle *et al.,* 2008). Excessive free radical production causes peroxidation of lipids, proteins and nucleic acids. Reoxygenation during reperfusion causes an increase in oxygen to levels that cannot be utilized by the mitochondria under normal physiological conditions. Thus, there is perturbation of the antioxidant defense mechanisms as a result of overproduction of oxygen radicals, inactivation of detoxification systems, consumption of antioxidants and failure to replenish antioxidants in the ischemic brain tissue (Kuroda and Siesjo, 1997; Doyle *et al.,* 2008). The compromised state of cellular defense mechanisms induces the production of free radicals that leads to oxidation of lipids, proteins and nucleic acids and finally, alters the cellular function in a critical way (Lewen *et al.,* 2000).

There are evidences that ischemic stroke triggers inflammatory reaction that is involved in late onset of symptoms and neuronal injury (Doyle *et al.,* 2008). Although, the exact mechanism of inflammation that contributes to ischemic damage is not well understood, altered microcirculation after ischemia/reperfusion leads to disruption in the blood-brain barrier, edema and swelling of perivascular astrocyte, decrease in arteriole endothelium-dependent relaxation and reduced inward-rectifying potassium channel function, altered expression of proteases and matrix metallo-proteinases, increased inflammatory mediators and inflammation are well reported (Takahashi and Macdonald, 2004; McCombe and Read, 2008). In the present investigation, OGD induced elevated levels of PGE_2 are also indicative that PC12 cells growing in the experimental setup have responded in the same fashion as in case of ischemic stroke. These observations are well in accordance with earlier studies using different *in vitro* (Tabakman *et al.,* 2002; Ahmad *et al.,* 2008) and *in vivo* (Farias *et al.,* 2008) experimental models of cerebral stroke

Inflammation related enzymes like nitric oxide synthase (iNOS) and cyclooxygenase (COX) are critical mechanisms by which inflammatory cells influence the progression of cerebral ischemic damage. In the brain, iNOS is induced in post ischemic inflammation, after transient or permanent middle cerebral artery occlusion in rodents (Iadecola *et al.,* 1995; Ross and Iadecola, 1996; Basu *et al.,* 2005). The increase in iNOS is associated with increases in iNOS enzymatic activity and nitric oxide production as revealed by accumulation of nitric oxide (Hirabayashi *et al.,* 2000; Dohare *et al.,* 2008). The presence of iNOS in human brain after ischemic stroke has

also been reported using immunohistochemical localization (Forster *et al.,* 1999; Murphy and Gibson, 2007). The data with iNOS inhibitors in null mice have suggested that the nitric oxide produced by iNOS is an important factor in the delayed progression of ischemic damage and can be targeted (Murphy and Gibson, 2007, Doyle *et al.,* 2008). In experimental model using PC12 cells, the levels of nitric oxide (NO) following OGD-reoxygenation insult were found to be increased, an observation that supports the usefulness of the measurement of NO levels as one of the endpoint. Since elevated levels of NO are also thought to be involved in oxidative stress induced cytotoxicity via diversified mechanisms (Forster *et al.,* 1999; Murphy and Gibson, 2007), the increased levels of NO in the studies with significant alterations in oxidative stress markers appear to be interlinked with each other as a part of cascade of events in pathophysiology of ischemic cerebral stroke.

Cytosolic Ca^{2+} rises as a result of net increase in the entry of Ca^{2+} across the plasmalemma or transiently due to liberation of Ca^{2+} from intracellular stores. In ischemia, an early increase in total cellular Ca^{2+} is also reported via NMDA receptors. This early influx of Ca^{2+} via NMDA receptors ceases, probably due to the profound ATP decrease and resulting receptor dephosphorylation and is replaced by influx through other pathways or release from intracellular stores (Lobner and Lipton, 1993). Under this experimental setup, PC12 cells have shown more than three fold increase in the accumulation of Ca^{2+} following 6 h of OGD and 24 h of reoxygenation. Thus, the results obtained in the present investigation are suggestive that an increased intracellular Ca^{2+} during ischemic stroke is, in part, responsible for cellular damage and intracellular Ca^{2+} levels could be an experimental biomarker of choice.

Studies demonstrated that the mitochondrial membrane potential increased when measured immediately after 6 h of OGD insult. These values recovered to the level of normoxia control after 24 h of reoxygenation. Therefore, mitochondrial membrane potential (MMP) appears to be an unlikely candidate as a biomarker to screen the anti-stroke potential of drugs using the *in vitro* model of ischemic stroke since, to commensurate with the clinical situations of stroke patients, the aim is to develop an *in vitro* model having the stable markers even after a sufficient reoxygenation period along with OGD. Iijima *et al.,* (2003) have also studied the levels of MMP in primary cultures of neuronal cells isolated from rat hippocampus following 30, 60 and 90 min OGD followed by 30 to 120 min of reoxygenation. They

also could not arrive at a firm conclusion since the levels of MMP were not following any specific pattern. In another study using PC12 cells, it was observed that MMP is directly proportional to the magnitude of oxidative stress induced by acrolein insult (Luo *et al.,* 2005). Shimizu *et al.,* (1996) have demonstrated a variable response for MMP in two different cell types i.e. CA1 pyramidal cells and granule cells in rat hippocampal slices presumably due to difference in types and density of ion channels and their distribution on the membranes (differential electrical properties of the cell membrane) even under the identical experimental conditions of OGD and reoxygenation.

In the nervous system, genes have been identified which either (i) promote apoptosis: Bax, c-fos, c-jun and ICE-like proteases, or (ii) block apoptosis: Bcl-2 and Bcl-xL. These genes that either suppress or augment cell death are expressed at higher levels and are activated in both the early and late phases of ischemia (Mattson *et al.,* 2000). Thus, in the present study, expression profile of these pro-apoptotic genes have been carried out and found to be responsive significantly to ischemic insult in cultured PC12 cells. Interestingly, besides the increase in the expression of pro-apoptotic genes, a decrease in the expression of Bcl-2 gene that is involved in anti-apoptosis could also be elicited. This indicates that the genes involved in both pro and anti apoptotic pathways are being activated and fully functional following ischemic insult in PC12 cells. Similar simultaneous activation of genes involved in pro and anti apoptotic pathways has been shown in a variety of cell systems including SH-SY5Y (Moran *et al.,* 2008), Neuro2a (Wu *et al.,* 2007), MCF-7 (Siddiqa *et al.,* 2008), HeLa (Jayaraj *et al.,* 2009) and LNCaP (Moon *et al.,* 2009).

Significantly high decrease in the dopamine DA-D_2 receptor levels could be detected in PC12 cells, which might be due to either the reduced number of viable cells (as evidenced by cytotoxicity endpoints carried out in the study) or a suppression in the expression of the receptor molecules on the cell surface. It has been documented that during ischemic insult, dopamine gets accumulated in the cells, which results in a decrease in the receptors (Wang *et al.,* 2002; Nader *et al.*, 2008; Son *et al.*, 2010). On the contrary, a number of studies have shown decreased level of dopamine in the cells subjected to ischemic insult. This may be due to either decreased synthesis (Lust *et al.,* 1975) or over secretion (Lavyne *et al.,* 1975; Weinberger, 2002) of dopamine. However, it is well evident now that both *in vivo* and *in vitro* studies that dopaminergic

neurons at substantia nigra region are associated with the hypoxic/ischemic conditions and undergo degeneration (Singh *et al.,* 2007).

Evaluation of anti-stroke potential of *trans* resveratrol using PC12 cells-OGD *in vitro* model of cerebral stroke

Resveratrol, a polyphenolic compound found in juice, wine from dark-skinned grape cultivars, nuts, pomegranates, and *Polygonum cuspidatum*, a component of Chinese herbal medicines (Leonard *et al.,* 2003) has been proven to possess anti-inflammatory, immunomodulatory, chemopreventive, neuroprotective and cardioprotective properties (Gusman *et al.,* 2001, Zhuang *et al.,* 2003). It is also synthesized by several plants in response to adverse conditions such as environmental stress or pathogenic attack (Baur *et al.,* 2006). For this reason, it is classified as a phytoalexin, a class of antibiotics of plant origin (Parker *et al.,* 2005; Valenzano *et al.,* 2006). One of the most interesting properties of resveratrol is its ability to confer potent neuroprotection in several models of brain injury, both *in vitro* (Zhuang *et al.,* 2003; Hans *et al.,* 2004) and *in vivo* (Wang *et al.,* 2002; Wang *et al.,* 2004; Huang *et al.,* 2001; Tsai *et al.,* 2007).

In the present investigations, the anti-stroke potential of *trans* resveratrol at three non-cytotoxic concentrations with pre, post and whole-treatment schedules to ascertain the prophylactic, therapeutic and synergistic applicability under clinical conditions of stroke have been studied. In general, a dose dependent protective effect could be observed under both pre and whole-treatment groups. A synergistic response was observed in whole-treatment group that might be due to a cumulative response of pre and post-treatment in whole-treatment group. Wang *et al.,* (2002) have demonstrated that chronic prophylactic treatment with *trans* resveratrol reduces the stroke-induced brain damage in experimental animals. The potent ability of *trans* resveratrol has also been demonstrated in reducing cerebral damage after ischemia/hypoxia (Wang *et al.,* 2002; Huang *et al.,*2001; Zamin *et al.,* 2006) trauma (Ates *et al.,* 2007), excitotoxicity (Wang *et al.,* 2004) and other conditions leading to neuronal demise (Kiziltepe *et al.,* 2004; Kumar *et al.,* 2006). The potential of prophylactic treatment may be of immense use for patients who have a family history of ischemic cerebral stroke or have suffered transient ischemic attack in the past.

Another reason for the better efficacy of *trans* resveratrol in whole-treatment group might be attenuation of free radical generation and elevation of antioxidant

defense mechanism in the cells. Similar findings of improved antioxidant status by pre-treatment with various antioxidant drugs including *trans* resveratrol has already been reported using *in vivo* experimental models (Slemmer *et al.,* 2008; Warner *et al.,* 2004).

In case of intracellular calcium, it seems that either an additional period of pre-treatment or higher concentrations of *trans* resveratrol might have a greater neuroprotective effect since the highest concentration (25μM) used in whole and post-treatment groups was able to bring down the Ca^{2+} values to basal level. In case of MMP, a drastic decrease was observed in OGD group immediately following the insult, which returned to basal level within 24 h of reoxygenation period. At this point of reoxygenation, values of all the treatment groups were almost parallel to the OGD and normoxia control. Therefore, under these circumstances, the affectivity of *trans* resveratrol is questionable and unexplained since the values of OGD group were also restored to basal level by self-recovery. Such a fast self-recovery in MMP precludes any utility of this endpoint as a biomarker for cerebral ischemia using PC12 cells-OGD *in vitro* model. Besides, MMP has also been found to be influenced by the physiological activity of cells (Shimizu *et al.,* 1996), time period of OGD and reoxygenation (Iijima *et al.,* 2003) and magnitude of oxidative stress following the external stimuli to the cells (Luo *et al.,* 2005). The experimental findings for PGE_2 are in agreement with the studies conducted in past using both *in vitro* and *in vivo* (Eduardo *et al.,* 2007) models demonstrating the strong anti-inflammatory response of *trans* resveratrol.

In the present study, all the treatment schedules and concentrations used were found to significantly reduce PGE_2 levels in PC12 cells that have received 6 h of OGD and 24 h of reoxygenation. However, no specific synergism has been observed for PGE_2 in whole-treatment group. This might be because the pre-treatment group itself brought the values to the normoxia level due to which any apparent response of synergism was insignificant in post and whole-treatment groups.

The present findings confirmed that OGD-reoxygenation insult reduces DA-D_2 bindings significantly as compared to normoxia control. Unlike the other endpoints studied, only pre-treatment of *trans* resveratrol was found to restore the DA-D_2 receptor bindings in a dose dependent manner, whereas no recovery could be seen in case of whole-treatment group. It is possible that pre-treatment of cells with *trans*

resveratrol enhanced their antioxidant status. Low amount of ROS generation eventually leads to low level of oxidative stress induced cellular damage including capability of DA-D_2 receptors to remains physiologically functional. Various other workers have also showed similar association of DA-D_2 receptors with ischemic insult. Saulle *et al.* (2002) have demonstrated dopamine induced neuronal injuries during ischemic stroke using electrophysiological recordings of neurons isolated from striatum region. Wang *et al.,* (2002) observed that during ischemic insult, dopamine gets accumulated in the cells, which results in decrease in the dopaminergic receptors. On the contrary, a number of studies have shown decreased amount of dopamine in the cells possibly due to a decrease in synthesis (Lust *et al.,* 1975) or over secretion (Lavyne *et al.,* 1975; Weinberger, 2002). In an isolated report Araki *et al.,* (1997) have hypothesized that the DA-D_2 receptors in the neurons of striatum region are particularly resistant to ischemic injury and not associated directly to the hypoxia induced damages in the brain. The protective potential of *trans* resveratrol observed in the present study following pre-treatment may also be explained on the basis of an indirect effect by decreasing the OGD induced-ATP consumption in the cells as a result of improved cellular physiological status and antioxidant capabilities (Guatteo *et al.,* 2005; Singh *et al.,* 2007). The lack of response in whole-treatment group or low response in post-treatment group of *trans* resveratrol may be due to the availability of very low number of physiologically functional cells after OGD-reoxygenation insult. Considering the mitochondrial activity and LDH release, it is well apparent that cells were under physiological stress (cytostatic response) in post and whole-treatment group. Under such situation, the available receptors might either be distorted or non receptive or may be very low in number.

Expression studies (mRNA and protein) were conducted to study the induction of pro and anti-apoptotic pathways following OGD-reoxygenation induced damage in PC12 cells. Interestingly, a significant increase in the mRNA expression was observed in all genes (c-Fos, c-Jun, Bax, GAP-43) involved in the process of apoptotic cell death and reduction in the expression of Bcl-2 gene (anti-apoptotic). The expression of genes involved in induction of apoptosis was found to decrease significantly and reached to almost basal level following whole and pre-treatment of *trans* resveratrol. In post-treatment group, significant changes in mRNA expression was observed. Although, the expression of immediate early response genes c-Fos and c-Jun have

been demonstrated in numerous *in vivo* experiments (Kinouchi *et al.,* 1994; Neumann-Haefelin *et al.,* 1994), only few reports are available demonstrating the expression c-Fos, c-Jun, GAP-43 after *in vitro* ischemia in cell culture system (Prabhakar *et al.,* 1995). In one study, PC12 cells were found to be a non-responder for c-Fos and c-Jun at transcription level against a noncompetitive NMDA receptor antagonist when compared with rat neuronal cultures (Gerlach *et al.,* 2002). Perhaps, this is the first report highlighting the induction of mRNA expression of pro-apoptotic genes and reduction in anti-apoptotic genes in PC12 cells following OGD and reoxygenation insult and its restoration to normoxia control using *trans* resveratrol. In a recent report, naloxone, a synthetic compound, has been reported to have the capability to reduce the elevated levels of c-Fos and c-Jun in *in vitro* model of ischemic stroke (Huang *et al.,* 2008).

Contrary to the transcriptional (mRNA) changes, expression at translational (protein) level was not found to recover significantly for any of the pro and anti apoptotic gene studied under the present investigation. However, the magnitude of recovery in whole-treatment group was greater than the pre and post-treatment groups. These findings suggest that transcriptional changes in the genes studied from the pro and anti-apoptotic pathways can be translated into cellular proteins. However, the effect of *trans* resveratrol treatment does not appear to be directly responsible for triggering off the genes responsible for their activation. This might be explained by the findings of Zhu *et al.,* (2004) who have demonstrated the expression of 76 predominant genes in hypertensive tissues of rat model of ischemic stroke and their role in the progression of disease through separate pathophysiological mechanisms at pre transcriptional, transcriptional and post-transcriptional levels.

Evaluation of anti-stroke potential of curcumin using PC12 cells-OGD *in vitro* model of cerebral stroke

Curcumin, a natural phenolic compound obtained as a major constituent in the rhizome of plant *Curcuma longa*, is being used as spice and medicine since time immemorial in Indian and Chinese system of medicine. It is a herbal medicine for which extensive scientific literature is available regarding its pharmacological efficacy as an antitumor, antioxidant, anti-inflammatory, anti-angiogenic and immunomodulator activities (Ozaki, 1990; Gonda *et al.,* 1993; Khar *et al.,* 1999; Maheshwari *et al.,* 2006; Daya *et al.*, 2008; Aggarwal *et al.,* 2009). In the present

investigation, the anti-stroke potential of curcumin at three non-cytotoxic concentrations with pre, post and whole-treatment schedules to ascertain its prophylactic, therapeutic and synergistic applicability under clinical conditions of stroke were studied. With an exception of one or two endpoints, pre-treatment of curcumin was found to be better than other treatment schedules followed by whole-treatment group. The post-treatment group was observed to have minimum restorative potential under the experimental set up. The antioxidant and anti-inflammatory activity of curcumin is well established in varieties of *in vitro* (Chan *et al.,* 1998; Luo *et al.,* 1999), *in vivo* (Rajakrishnan *et al.,* 1994; Zhou *et al.,* 2004; Daya *et al.*, 2008) and clinical studies (Ringman *et al.,* 2005). It is also reported that prophylactic treatment of curcumin has shown better protective potential than therapeutic interventions in various neurodegenerative disorders (Cole *et al.,* 2007) including transient cerebral stroke (Wang *et al.,* 2005b; Shukla *et al.,* 2008). In a similar fashion, pre-treatment of many other antioxidants/anti-inflammatory drugs has also been demonstrated to have better protective potential than therapeutic recovery in experimental models of cerebral stroke (Gupta *et al.,* 2002b; Chaudhary *et al.,* 2003a, 2003b; Briyal *et al.,* 2007) and other neurodegenerative disorders (Kumar and Gupta, 2003; Sharma and Gupta, 2002, 2003; Sharma *et al.,* 2005; Ramassamy, 2006). However, there was no response of any treatment schedule of curcumin on the restoration of lipid peroxidation. This can be correlated with the enhanced mitochondrial activity due to excessive availability of intracellular oxygen during reoxygenation period, which is known to lead cytotoxic responses in the cell (Salvioli *et al.,* 2007). In this investigation, enhanced mitochondrial activity could be recorded in pre and post-treatment groups (cytostatic response) with severe cytotoxic responses in whole-treatment group.

Curcumin treatment was found to have a detrimental effect on SOD activity and further decreased its level when compared with OGD and normoxia groups especially in the post-treatment group. A synergistic response of curcumin was evident in the whole-treatment group probably due to an enhancement of the antioxidant and anti-inflammatory status of the cells. However, in post-treatment group, the antioxidant status of the cells was comparatively lower than the pre-treatment group due to which they were more vulnerable to OGD-reoxygenation insult and exhibited maximum detrimental activity for SOD activity among all the treatment groups. This detrimental effect of curcumin on SOD activity may be due to the activation of genes

involved in the apoptotic pathways (Tsubokawa *et al.,* 2007) as enhanced expressions of pro apoptotic genes and down regulation of anti apoptotic genes was also observed in the present investigations.

In general, whole-treatment group exhibited lower restoration potential than pre-treatment group in most of the endpoints studied. Paradoxically, the higher doses showed less efficiency/detrimental effects in whole-treatment group as compared to groups receiving lower doses. Though curcumin is well known for its anti-inflammatory and antioxidant activities, at the same time, its pro-oxidant activity at higher doses and long exposure period has also been reported in various experimental models (Cole at al, 2007; Salvioli *et al.,* 2007). Thus, a long exposure of 52 h (24 h pre-treatment + 6 h during OGD + 24 h during reoxygenation) of curcumin at concentrations of 25, 50 and 100 μg/ml may be considered as chronic exposure of higher concentrations of curcumin for the PC12 cells-OGD *in vitro* model of ischemic stroke. Interestingly, the cells kept under normoxia conditions exposed to these concentrations of curcumin for 52 h were found with a healthy antioxidant status, while in the cells kept under whole-treatment group, impairment in antioxidant status and mitochondrial activity could be demonstrated. The genes involved in apoptotic pathways are known to get activated during OGD insult (Takman *et al.,* 2004; Jiang *et al.,* 2005; Koubi *et al.,* 2005), which could be a reason for the decrease in efficacy of curcumin or reversal of its effect.

The level of intracellular calcium were restored to near normoxia control with the lowest concentration of curcumin used for pre-treatment while higher doses in the same group can be considered as pro oxidant since the highest concentration of curcumin used in the study was found to have pro apoptotic/pro-oxidant response in many of the studied endpoints. At higher doses, curcumin has also been reported to cause ROS generation mediated elevation of cytosolic calcium through the release of calcium ions from intracellular stores as well as by influx of extracellular calcium and subsequent cell death in *Leishmania donovani* (Das *et al.,* 2008). Similar findings were also observed in colon carcinoma cells (Colo205) (Su *et al.,* 2006). Cao *et al.,* (2006, 2007) have demonstrated that lower doses of curcumin did not produce oxidative stress in HepG-2 human hepatoma cell lines whereas higher concentrations are potentially involved in ROS generation mediated cytotoxicity and genotoxicity in these cells.

Further, in case of mitochondrial membrane potential (MMP), a drastic decrease was observed in OGD group immediately after OGD, which come down to basal level by 24 h of reoxygenation period. At this point of reoxygenation, values of all the treatment groups were almost parallel to the OGD and normoxia control, as in case of *trans* resveratrol. Therefore, under these circumstances the affectivity of curcumin is questionable and unexplained, since the values of OGD group also came down to basal level by self-recovery. As the findings for MMP for both the drugs used in the study are similar and at par to normoxia and OGD group, it is evident that both the drugs used in the study have no adverse effect on the MMP levels at any concentration. Though the self-recovery reached the normoxia level by 24 h of reoxygenation, such a fast self-recovery in MMP suggests no utility of this endpoint as a biomarker to study drug intervention using this model. Moreover, MMP was found to be affected by physiological activity of cells (Shimizu *et al.,* 1996), time period of OGD, re-oxygenation (Iijima *et al.,* 2003) and magnitude of oxidative stress also (Luo *et al.,* 2005).

Like *trans* resveratrol, all the treatment schedules with curcumin were also found to be effective for the control of OGD-induced alterations in PC12 cells. However, no adverse affect of higher doses could be detected and an almost equal magnitude of reduction in PGE_2 levels was recorded. These findings are almost parallel to the earlier findings for a variety of experimental setups using different cell/tissue systems following toxic insults and restoration by curcumin (Moon *et al.,* 2005; Lev-Ari *et al.,* 2006a,b; Park *et al.,* 2007; Hsieh *et al.,* 2008; Huang *et al.,* 2008). Extended exposure of 52 h of curcumin did not elicit any additive response in whole-treatment group. Experimental findings for PGE_2 are in well accordance with the earlier studies using both *in vitro* (Moon *et al.,* 2005; Lev-Ari *et al.,* 2006a,b; Hsieh *et al.,* 2008) and *in vivo* (Park *et al.,* 2007; Huang *et al.,* 2008) models demonstrating the strong anti-inflammatory response of curcumin.

Treatment with both curcumin and *trans* resveratrol exhibited similar changes in DA-D_2 receptor levels. OGD-reoxygenation insult causes a highly significant reduction in DA-D_2 receptor binding in PC12 cells receiving OGD of 6 h and reoxygenation of 24 h. In pre-treatment group, a dose dependent recovery could be seen followed by post-treatment and whole-treatment. It appears that better antioxidant status due to pre-treatment with curcumin decreases the amount of ROS generation

and retains the physiological functionality of DA-D_2 receptors. The association of DA-D_2 receptors with ischemic insult has been shown by various workers. Saulle *et al.,* (2002) have demonstrated dopamine induced neuronal injuries during ischemic stroke using electrophysiological recordings of neurons isolated from striatum region. Wang *et al.,* (2002) have demonstrated that during ischemic insult, dopamine gets accumulated in the cells, which results in a decrease in the receptors. On the contrary, a number of workers have shown decreased amount of dopamine in the cells either due to low amount of synthesis (Lust *et al.,* 1975) or over secretion (Lavyne *et al.,* 1975; Weinberger, 2002). The protective potential of curcumin following pre-treatment may be as a result of decrease in OGD-induced ATP consumption by the cells by improving their overall physiological status and antioxidant capabilities (Guatteo *et al.,* 2005; Singh *et al.,* 2007). The absence of any significant response with curcumin in the whole-treatment group or low response in post-treatment group may be attributed to a decrease in the number of physiologically functional cells after OGD of 6 h and reoxygenation of 24 h. The results of studies on mitochondrial activity and LDH release assay support the contention that cells were under physiological stress (cytostatic response) in post and whole-treatment group due to which the receptors available might be either distorted or non receptive and above all very low in number.

Expression studies (mRNA and protein) were conducted to study the induction of pro and anti-apoptotic pathways following OGD-reoxygenation induced damages in PC12 cells. Both curcumin and *trans* resveratrol exhibited similar effects on the expression of mRNA. In case of curcumin also, mRNA expression for all the genes involved in the apoptosis induction decreased significantly and reached the basal level. Induction in mRNA expression for immediate early response genes i.e. c-Fos, c-Jun and GAP-43 have also been reported in cultured neuronal cells following various chemical induced toxic insults including PC12 cells (Seth *et al.,* 2002; Huang *et al.,* 2008). However, the changes induced in mRNA expression in c-Fos, c-Jun and GAP-43 genes and restoration by curcumin and *trans* resveratrol in PC12 cells following OGD-reoxygenation insult has never been studied.

Contrary to the transcriptional (mRNA) changes, expressions at translational (protein) level were not found to have recovered significantly for any of the pro and anti apoptotic gene studied under the present investigation. However, the magnitude of recovery in pre-treatment group was slightly greater than the post and whole-treatment

groups. These findings are suggestive that the transcriptional changes in the genes studied from the pro and anti apoptotic pathway could be translated into cellular proteins. However, the effect of curcumin treatment was not directly responsible for triggering of the genes responsible for their activation. This might be explained by the findings of Zhu *et al.,* (2004) who have demonstrated the expression of 76 predominant genes in hypertensive tissues of rat model of ischemic stroke and their role in the progression of disease through separate pathophysiological mechanisms at pre-transcriptional, transcriptional and post-transcriptional levels. The association and control of many of the anti- and pro-apoptotic pathways is now established through various post-translational factors (Ashkenazi *et al.,* 1998; Wang *et al.,* 1998; Henis-Korenblit *et al.,* 2000) that are responsible to control the physiological activities of many proteins after translation. It is possible that the genes related to pro and anti apoptotic pathways selected in the present study may be regulated through post-translational factors in PC12 cells receiving OGD insult.

On the basis of these observations on the anti-stroke potential activity of *trans* resveratrol and curcumin in PC12 cells receiving OGD of 6 h and reoxygenation of 24 h, it could be concluded that PC12 cells-OGD *in vitro* model of cerebral stroke mimics most of the endpoints occurring during stroke and responds specifically for both the test drugs. It can be a model of choice for *in vitro* screening of anti-stroke potential of new molecules. Both *trans* resveratrol and curcumin were found to have significant anti-stroke potential as evident by restoration of various endpoints involved in the cascade of events during ischemic stroke, whole-treatment with *trans* resveratrol and pre-treatment with curcumin were found to be better than other treatments. *Trans* resveratrol was selected for *in vivo* validation studies in rat MCAo model of cerebral stroke, since studies have already been carried out with curcumin.

Evaluation of anti-stroke potential of curcumin in rat MCAo model of cerebral stroke

To study the downstream responses of cellular changes in physiological functionality of the brain, *in vivo* studies were conducted using rat MCAo model of cerebral stroke. In order to see the extrapolation of *in vitro* data with *in vivo* system, *trans* resveratrol was used in pre, post and whole-treatment groups corresponding to the treatment schedules for transient as well as for the onset of ischemic cerebral stroke. Anti-stroke potential of *trans* resveratrol was mainly assessed by its antioxidant

activity, restoration of DA-D_2 receptor binding capability and neurobehavioral endpoints since, oxidative stress mediated damages and impairment of sensory motor functions are well known events involved in the pathophysiology of cerebral stroke and correlate well with ischemic damage (Markgraf *et al.,* 1992; Rogers *et al.,* 1997).

Data confirmed that ischemic insult following 24 h and 7 days of reperfusion was capable of inducing neurological damage in rat MCAo model. Self-recovery was also reported by 7 days of reperfusion without any drug treatment. Pre-treatment with *trans* resveratrol was found to be the best amongst the treatment groups in reducing the neurological deficits (in terms of neurological scores) in both 24 h and 7 days of reperfusion periods. Neurological score correlates well with severity of infarction (Rogers *et al.,* 1997; Ding *et al.,* 2002). Grip strength is one of the sensitive tests to monitor the muscle strength. Rats with ischemic reperfusion injury following transient and permanent occlusion of middle cerebral artery have been reported to show impairment in grip strength (Rogers *et al.,* 1997). Highly significant reduction in the forelimb grip strength was recorded in ischemic rats with a marginal self-recovery in extended reperfusion period of 7 days. Like neurological scores, in grip strength also, pre-treatment of *trans* resveratrol was found to be most effective especially for animals with 7 days of reperfusion. Spontaneous locomotor activity is one among the most widely used test to assess the changes in the exploratory behaviour and muscle coordination following ischemic stroke (Chaudhary *et al.,* 2003a, b; Shukla *et al.,* 2008). On the other hand, some investigators have reported no specific and significant relevance of spontaneous locomotor activity in focal ischemia (Borlongan *et al.,* 1995; Wood *et al.,* 1996; Yonemori *et al.,* 1999). Few reports suggest spontaneous locomotor activity as an insensitive and mis-guiding sensory motor function to evaluate the ischemic insult induced responses (Grabowski *et al.,* 1991; Hunter *et al.,* 2000; Reglodi *et al.,* 2003; Wang *et al.,* 2005; Chen *et al.,* 2006). In the present investigations, the trend in the alteration of spontaneous locomotor activity because of ischemic insult and recovery pattern following treatment of *trans* resveratrol were in accordance with neurological deficits and grip strength.

Enhanced oxidative stress due to increased generation of free radicals has been reported during cerebral ischemia (Callaway *et al.,* 1999; Kinouchi *et al.,* 1991; Pierre *et al.,* 1999; Chaudhary *et al.,* 2003a, 2003b). An increase in the levels of oxygen and hydroxyl radicals following MCAo has also been shown (Ghoneim *et al.,* 2002).

However, free radical generation is enhanced more during reperfusion (Callaway *et al.,* 1999). Kuroda and Siesjo, (1997) suggested that generation of reactive oxygen species is an important contributor to brain damage. An increase in lipid peroxidation and decrease in superoxide dismutase activity and glutathione levels in brain of animals receiving 30 min MCAo followed by reperfusion of either 24 h or 7 days observed in the present study suggest a state of enhanced oxidative stress. Protective effect of *trans* resveratrol against cerebral ischemia in rats has also been reported through its antioxidant property, when administered intravenously (Jean-francois *et al.,* 2002). However, in the present investigation, *trans* resveratrol was administered through clinically relevant oral route. Oral route of administration was selected since it is a convenient route of administration under clinical situations and has been found to have better bioavailability and pharmacokinetic properties over intraperitoneal routes.

The decrease in body weight following MCAo observed in the present study is consistent with the earlier reports of post-ischemic loss in body weight (Rogers *et al.,* 1997; Callaway *et al.,* 1999; Shukla *et al.,* 2008). Gracia and Liu, (1996) reported that the body weight decrease was probably due to infarction affected feeding behaviour and injury to the anterior hypothalamus. The decrease in body weight in ischemic rats in the present study could be due to decreased food intake of these rats. Since multiple factors are involved in ischemic damage, large numbers of synthetic and natural neuroprotective agents have been investigated to study their anti-stroke potential (Curtis-Prior *et al.,* 1999; Pierre *et al.,* 1999). Vitamin B_3 and E, NMDA receptor antagonists, Na^+ channel blockers and nitric oxide synthase (NOS) inhibitors have been used and found effective in experimental models of stroke (Chabrier *et al.,* 1999; De Keyser *et al.,* 1999; Mokudai *et al.,* 2000). Use of herbal products in the management of ischemia has also been advocated because of their high antioxidant activity and low side effects (Curtis-Prior *et al.,* 1999; Pierre *et al.,* 1999). Dietary supplementation with blueberries, spinach and spirulina have been reported to reduce ischemia/reperfusion induced apoptosis and cerebral infarction (Wang *et al.,* 2005a). It has been reported that agents that inhibit lipid peroxidation or have strong antioxidant activity are useful in the treatment of ischemic conditions (Curtis-Prior *et al.,* 1999; Pierre *et al.,* 1999; Huh *et al.,* 2000). Neuroprotective potential of BN 80933, a dual inhibitor of lipid peroxidation and NOS has been reported in different experimental models of cerebral stroke (Chabrier *et al.,* 1999). Besides its strong antioxidant activity

resveratrol plays a role in the prevention of human pathological processes, such as inflammation (Jang *et al.,* 1999), atherosclerosis and carcinogenesis (Jang *et al.,* 1997).

The present study has demonstrated, using both *in vitro* and *in vivo* approaches that these properties of *trans* resveratrol might have contributed to its anti-ischemic efficacy. The protective effect has been attributed to its antioxidant properties (Jang *et al.,* 1999), to an anticyclooxygenase activity (Jang *et al.,* 1997), and to a modulating activity of lipid and lipoprotein metabolism (Goldberg *et al.,* 1995). Resveratrol also inhibits platelet aggregation and exhibits antiestrogenic activity (Lu R *et al.,* 1999; Gehm *et al.,* 1997). It was observed in the present investigations that pre-treatment of *trans* resveratrol was more effective in reducing lipid peroxidation, and increasing the super oxide dismutase, catalase activity and glutathione levels in the cells at both 24 h and 7 days of reperfusion periods. Leonard (2003) has also observed that resveratrol exhibits a protective effect against lipid peroxidation in cell membranes and DNA damage caused by ROS.

Interestingly, a significantly lower magnitude of antioxidant activity of *trans* resveratrol in post-treatment schedules was observed. Further, after multiple doses of post-treatment i.e. for 7 days during reperfusion period, *trans* resveratrol was found to be causing adverse or no effects as compared to beneficial effect of lower dose. These findings are well in accordance with the infarction volume observed in the present investigations. The poor performance of post-treatment group might be explained due to marked impairment in the antioxidant status of the animals by ischemic insult along with damages induced by reactive oxygen species during reperfusion. In the pre-treatment group animals were found capable enough to overcome such damages as *trans* resveratrol exposure prior to ischemic insult is supposed to elevate the antioxidant status in the animals.

The maximum tolerated dose of resveratrol has not been thoroughly determined, but 300 mg per kg (body weight) showed no detrimental effects in rats (Crowell *et al.,* 2004) and doses up to 100 mg per kg (body weight) have been used routinely in studies on rodents. Resveratrol has a short initial half-life ~8–14 min for the primary molecule (Marier, *et al.,* 2002; Asensi *et al.,* 2002) and is metabolized extensively in the body. A 28-day study of the effects of 20 mg per kg (body weight) oral resveratrol in adult rats found no effect on body weight, food or water

consumption, hematological or clinical biochemistry variables, or histopathology (Juan *et al.,* 2002), and no adverse effects were observed at doses of up to 300 mg per kg (body weight) (Crowell *et al.,* 2004).

In addition to its other properties, resveratrol is reported to act as an analgesic (Gentilli *et al.,* 2001; Granados-Soto *et al.,* 2002), protect against hearing loss (Seidman *et al.,* 2003) and enhance lipopolysaccharide-induced anorexia in rats, although it has no anorexic effect when given alone (Lugarini *et al.,* 2003). Resveratrol has also been shown to reduce injuries to the kidneys (Cadenas *et al.,* 1999; Giovannini *et al.,* 2001), spinal cord (Yang *et al.,* 2002; Yang *et al.,* 2003), liver (Fulgenzi *et al.,* 2001), lungs (McClintock *et al.,* 2002), intestine (Korolkiewicz *et al.,* 2003; Korolkiewicz *et al.,* 2004) and colon (Martin *et al.,* 2004). These additional results indicate that the protective effects of resveratrol are not limited to the heart and brain *in vivo*.

The antioxidant effect of resveratrol in the present study may be attributed to blood-brain barrier permeability as demonstrated in previous studies (Wang *et al.,* 2002; Mokni *et al.,* 2007). Relatively simple chemical structure enables *trans* resveratrol to interact with receptors and enzymes, giving rise to biological effects such as suppression of growth, induction of differentiation, inhibition of reactive oxygen intermediates production, cell cycle regulation, inhibition of lipid peroxidation, down-regulation of proinflammatory mediators, regulation of gene expression by affecting transcription factor activity, and upregulation of death-inducing factors (Pervaiz 2003).

In present investigations, increase/restoration in infarction volume is not so prominent and highly significant but the trend was well in coordination with observed behavioral and biochemical parameters in MCAo rats following pre, post and whole-treatment of *trans* resveratrol. In holistic view, data obtained through *in vitro* PC12 cells-OGD model have shown reasonably significant correlation and extrapolative values with the data generated using *in vivo* rat MCAo model of cerebral stroke under experimental set up.

These studies would be of immense significance not only in understanding the mechanisms involved in the ischemic cerebral stroke at cellular and molecular levels, but would also be useful in adopting this PC12 cells-OGD system as a cost effective, reliable, more sensitive with better predictive values for screening of anti-stroke

potential of drug candidate molecules in very rapid way. Further, this system would also be useful to extrapolate the data with rat MCAo model of ischemic stroke and to the clinical situations. Although, this piece of work is not a milestone in the prevention of ischemic stroke induced impairments and neuronal injuries, but is definitely a step towards reducing the suffering and agony of poor patients suffering with this fatal disease.

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PUBLICATIONS

1. **M Agrawal**, GS Randhawa and AB Pant. Standardization of factors associated with ischemic cerebral stroke in cultured rat pheochromocytoma cells. International Symposium on "Monitoring and Modulating Global Resources of Environmental and Food Contaminants: Nature versus Chemicals" and 28th Annual Conference of Society of Toxicology (STOX), 2008 at Department of Pharmacology & Toxicology College of Veterinary Science, Ludhiana, (India).

3. **M Agrawal**, V Kumar, MA Siddiqui, MP Kashyap, GS Randhawa and AB Pant. Validation of *in vitro* ischemic cerebral stroke model using *trans* resveratrol, a natural neuroprotective agent. International Symposium on "Current Trends in Toxicology & Pre-Clinical Toxicology" and 29th Annual Conference of Society of Toxicology (STOX), 2009 at National Institute of Nutrition, Hyderabad, (India).