Differential role of Sirtuin-1 following cerebral ischemia in rat and human

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DOCTOR OF PHILOSOPHY

By

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"**Differential role of Sirtuin-1 following cerebral ischemia in rat and human**"** has been carried out by me under the supervision of Prof. P. Prakash Babu in the Dept. of Biotechnology & Bioinformatics, School of Life Sciences, University of Hyderabad. This work has not been submitted for any degree or diploma of any other University or Institute.

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CERTIFICATE

This is to certify that the thesis entitled "Differential role of Sirtuin-1 following cerebral ischemia in rat and human" submitted by **T. Sireesh Kumar**, bearing registration number **11LTPH15** in partial fulfilment of the requirements for award of Doctor of Philosophy in the **Department of Biotechnology & Bioinformatics, School of Life Sciences** is a bonafide work carried out by him under my supervision and guidance.

This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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4	BT 804	Analytical Techniques	3	**Pass**
5	BT 805	Lab Work	4	**Pass**

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Abbreviations:

°C	degree Celsius
λ	wavelength
μ	micro-
2-VO	Two-vessel occlusion
4-VO	Four-vessel occlusion
AA	Arachidonic acid
AIF	Apoptosis inducing factor
AKT	Protein Kinase-B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APAF-1	Apoptotic protease activating factor-1
ASK-1	Apoptosis signal-regulating kinase-1
ATG	Autophagy related protein
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma-2
Bcl-xL	B-cell lymphoma extra large
BDNF	Brain-derived neurotrophic factor
Bid	BH3 interacting domain death agonist
CCA	Common carotid artery
COX	Cyclooxygenase
Cyt-c	Cytochrome-c
DAPI	4,6-diamidino-2-phenylindole
DRAM	Damaged-regulated autophagy modulator
ECA	External carotid artery
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
FADD	FAS-associated protein with death domain
FOXO	Forkhead box transcription factor
GFAP	Glial fibrillary acidic protein
GPX	Glutathione peroxidase
H&E	Hematoxylin-Eosin
HIF	Hypoxia-inducible factor
HNE	4-hydroxy nonenal
ICA	Internal carotid artery
ICAM	Intracellular adhesion molecule-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I/R injury	Ischemia-reperfusion injury

IRS-1	Insulin receptor substrate-1
JNK	C-Jun N-terminal kinase
LC-3	Microtubule-associated protein 1A/1B-light chain-3
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
MDA	Malondialdehyde
MEK	Mitogen-activated protein kinase kinase
MIR	MicroRNA
MMP	Matrix metalloproteinases
NAD	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NCX-3	Sodium-calcium exchanger-3
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NMDA	N-methyl D-aspartate receptor
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
p53	Tumor protein p53
PANX-1	Pannexin-1
PFA	Para formaldehyde
PBS	Phosphate-buffered saline
PI3K	Phosphoinositol-3 kinase
PUMA	p53 upregulated modulator of apoptosis
RNS	Reactive oxygen species
Sirt1	Silent mating type information regulation 2 homolog-1
SOD	Superoxide dismutase
TGF	Transforming growth factor
TIGAR	TP53 induced glycolysis regulatory phosphatase
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TRAIL	TNF related apoptosis-inducing ligand
TRP	Transient receptor potential channel
TTC	2,3,5-Triphenyl tetrazolium chloride
VCAM	Vascular cell adhesion protein-1
VPS	Vacuolar protein sorting-associated protein
XO	Xanthine oxidase

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तद्विद्धि प्रणिपातेन परिप्रश्नेन सेवया ।
उपदेक्ष्यन्ति ते ज्ञानं ज्ञानिनस्तत्त्वदर्शिनः ॥

Dedicated to my guide Prof. Prakash Babu

Nature doesn't ask your permission; it doesn't care about your wishes, or whether you like its laws or not. You're obliged to accept it as it is, and consequently all its results as well.
Fyodor Dostoevsky

Introduction:

Introduction:

Stroke is a devastating condition leading to human death and long-term disability. Globally 17 million people are affected by stroke every year in which 30-35% of stroke causes death. Another 10-15% of the affected individuals die during first year after attack (Benjamin et al. 2017), (Popa-Wagner et al. 2007), (Badan et al. 2003). Other survivors have various neurological consequences and become burden to family and society. Stroke can affect individuals of any age but is more prevalent in aged individuals. Age is one of the significant irreversible risk factor for stroke (Kelly-Hayes et al. 2003). Stroke is principally classified into two main categories: Ischemic and hemorrhagic stroke. The ischemic stroke occurs when an occlusion or embolism prevents blood supply to the brain, which results in insufficient oxygen and glucose delivery to support brain cell survival. Ischemic stroke accounts for 87% of the stroke cases. Hemorrhagic stroke occurs by rupture of arteries within the brain and it accounts for 15% of the stroke cases (Amarenco et al. 2009). The ischemic stroke is further subdivided into two types: focal and global ischemic stroke. A reduction in blood flow causes focal ischemia to a particular brain region. mainly to the territory of middle cerebral artery (MCA), which is the major cause of stroke in humans (Shiber, Fontane, and Adewale 2010a). A reduction in blood flow to the majority of the brain regions causes global ischemia and is encountered during cardiac arrest.

The ischemic cerebrovascular bed mainly comprises of two critical regions of the brain with distinct molecular mechanisms, the ischemic core and ischemic penumbra. The characteristic feature of the ischemic core is irreversible tissue damage with immediate necrotic cell loss due to sudden reduction in blood flow. The ischemic penumbra is the region of metabolically active tissue with diminished blood flow, which relies on the periphery to the core (Yu et al. 2016). Apoptosis is the programmed cell death mechanism prevailing in penumbra, which may occur after a few hours to days after ischemic insult (Broughton, Reutens, and Sobey 2009). Ischemic penumbra is characterized by the activation of harmful molecular mechanisms that trigger apoptotic cell death, including ATP depletion, an increase in oxidative stress, Ca^{2+} overload, spreading depolarization, and glutamate excitotoxicity (Durukan and Tatlisumak 2007). The delay in penumbral cell loss draws attention of researchers and clinicians to find effective therapeutic strategies to rescue from brain cell damage and prevention of ischemic infarct extension. Currently, tissue plasminogen activator (tPA), a thrombolytic agent, is the most suitable therapeutic interventions for AIC (acute ischemic stroke) within the small window period. The drawback with tPA treatment is that it can promote blood brain barrier permeability and intracerebral hemorrhage. Administration of tPA can also cause reperfusion-induced secondary injury, including oxidative stress, apoptosis, and inflammation, (Tobin et al. 2014). Thromboembolism is also known to improve stroke outcome in severe cases with large artery thrombosis.

Experimental animal models in stroke study:

In recent years the use of experimental animal models for stroke studies have provided more knowledge of the pathophysiology of stroke. The majority of the pre-clinical stroke studies are conducted in small experimental rodents like rats and mice with the growing use of larger species, such as rabbits and nonhuman primates. There are several different models of cerebral ischemia, each with strengths and constraints when mimicking the complex nature of the clinical stroke. The experimental rodent model, like the rat, is one of the most widely used animal in pre-clinical stroke studies. The cerebral circulation and anatomy of the rat are similar to that of humans. The smaller brain size is well suited for histological studies, and it is easy to conduct reproducible studies. On the other hand, the mouse is the most suitable animal for transgenic studies that evaluate the pathophysiology of stroke. The four-vessel occlusion (4-VO) model is well established for the induction of global ischemia, which mainly involves blocking of blood flow in both the carotid arteries and vertebral arteries for a specific time. Another well-known model for the induction of reversible global ischemia is two-vessel occlusion (2-VO), in which bilateral common carotid artery occlusion is coupled with systemic hypotension (Traystman 2003). The 2-VO is an alternative to the 4-VO for the induction of reversible forebrain ischemia. Human ischaemic stroke usually results from the occlusion of the MCA, and therefore MCA occlusion is the model employed in pre-clinical stroke studies. The intraluminal nylon monofilament model is the most commonly used model for the generation of focal cerebral ischemia. The intraluminal filament model can produce a considerable volume of potentially salvageable penumbra and makes this model useful for studying various therapeutic interventions (McCabe et al. 2018).

Pathophysiology of ischemic cell death:

The ischemic pathophysiology is the combined outcome of various molecular events involved in brain cell death, which are summarised below.

Excitotoxicity:

Glutamate is one of the most important neurotransmitter that involves in neuronal growth, synaptic plasticity, brain development and maturation. Ischemic injury can trigger an abundant release of excitatory neurotransmitter glutamate from the brain cells in the ischemic core region. Since brain cells in ischemic penumbra have insufficient ATP, they fail to reuptake glutamate via glutamate transporter (Zerangue and Kavanaugh 1996), which is an energy dependent transport system and ultimately the excessive glutamate released by brain cells in core will accumulate in extracellular space and can lead to the uncontrolled activation of ionotropic glutamate receptors, which are ligand-gated ion channels (Choi and Rothman 1990). The ionotropic glutamate receptors are central players in glutamate induced excitotoxicity, which mainly comprise of NMDA receptors (NMDAR), AMPA receptors (AMPAR), and kainate receptors (KR). The NMDAR are the key players in glutamate induced excitotoxicity, which are activated by glutamate and co-agonist glycine. The over stimulated NMDAR allow an influx of Ca^{2+} in to the cytosol and promotes the activation of various enzymes including neuronal nitric oxide synthase (nNOS) and the calcium activated protein calpain. The excess nitric oxide (NO) produced by nNOS and calpain contributes to mitochondrial dysfunction and opening of mitochondrial transition pore complex and promotes the release of cytochrome-c (cyt-c) and apoptosis –inducing factor (AIF) in to the cytosol there by promoting neuronal cell death (Potts et al. 2003), (Johnson et al. 2007), (D'Orsi et al. 2012). Further overstimulation of AMPAR and KR by extracellular glutamate can also increase excitotoxic cell death related to NMDAR (Gan, Salussolia, and Wollmuth 2015). In addition to ionotropic receptors, some of the plasma membrane proteins can also induce excitotoxic cell death by promoting influx of Ca^{2+} into cytosol. These proteins include sodium-calcium exchanger-3 (NCX-3), ion channel pannexin-1(panx-1), transient receptor potential channels (TRP), and sensing ion channels (ASKs), which are activated in response to the increased Ca^{2+} influx caused by over stimulated NMDA receptors (Szydlowska and Tymianski 2010).

Oxidative stress:

The ischemia-reperfusion (I/R) induced secondary injury promotes the activation of various molecular mechanisms. Among numerous complex molecular cascades activated after I/R injury, free radical damage to the brain plays a pivotal role in reperfusion-induced secondary injury (Yamato, Egashira, and Utsumi 2003). The brain represents 2% of the whole body weight and consumes 20% of the body's oxygen. The brain is more vulnerable to free radical damage as it comprises of high content of lipids with unsaturated fatty acids and free iron (Dringen 2000). Free radicals can be subdivided into two major groups: reactive oxygen species (ROS) and reactive nitrogen species (RNS). Under normal physiological conditions, ROS plays an essential role in the regulation of synaptic plasticity, immune response, apoptosis, and act as secondary messenger for signal transduction (Lei and Kazlauskas 2014), (Bae et al. 1997). Excess ROS is mainly generated from four signaling pathways, including mitochondrial respiratory chain (MRC), NADPH oxidase, Arachidonic acid produced by lipid peroxidation, xanthine and hypoxanthine production by Xanthine oxidase (Margaill, Plotkine, and Lerouet 2005). During the reasonable physiological condition, the detrimental type of ROS, including superoxide anion (. O_2^-), hydroxyl radicals (OH^-), and hydrogen peroxide (H_2O_2), were catalyzed by superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase in the brain cells (Yoshioka et al. 2002), (Robinson and Winge 2010). During I/R, ROS production increases significantly, which leads to the failure of the antioxidant defence system. The brain cells trigger the glycolytic pathway for ATP generation during hypoxia-ischemia, which leads to the production of lactic acid and H^+ in the mitochondrial lumen and the reversal of the H^+ uniporter on the mitochondrial membrane, which causes an increased production of H_2O_2 and hydroxyl radicals (Ying et al. 1999). The ROS produced by NADPH oxidase in the immune cells can act as a defence mechanism against various pathogens. During ischemia-reperfusion, NADPH oxidase in immune cells and vascular endothelial cells can produce excessive ROS and promote oxidative stress (Walder et al. 1997). The lipid peroxidation mediated by cyclooxygenase (COX) and arachidonic acid (AA) pathway activates the generation of various by-products, including malondialdehyde (MDA) and 4-hydroxynonenal (HNE), which induce apoptotic brain cell death(Matsuda et al. 2009). Xanthine oxidase (XO) is an essential source of superoxide radicals in rat after I/R injury (Ono et al. 2009). The stress-induced mitogen-activated protein kinases (MAPK), including JNK1/2 and p38, can be induced by an apoptosis-signal regulated kinase (ASK1), which is influenced by the excess ROS. Further constitutive activation of JNK1/2 is ROS dependent (Song et al. 2013), (Davis 2000). ROS can also induce p53 activation via cyclophilin-D, which opens mitochondrial transition pore and the release of Cyt-c and promotes apoptosis (Endo et al. 2006). The primary reactive nitrogen species (RNS) are nitric oxide (NO) and peroxynitrite ($ONOO^{-)}$. NO is mainly catalyzed by three types of nitric oxide synthases (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). The NO generated from eNOS promotes vasodilation, while NO produced from iNOS and nNOS is harmful. The excess NO generated by iNOS and nNOS can promote blood-brain barrier (BBB) breakdown via the activation of matrix metalloproteinases (MMP) and also enhances cyclooxygenase-2 activity and inflammation. $ONOO^-$ can also engage in the dysfunction of mitochondrial electron transport chain complex and MMP activation after stroke (Sun et al. 2018).

Inflammation:

Secondary injury after I/R contributes significantly to the development of post-stroke inflammation. Ischemia triggers the release of inflammatory mediators from necrotic cells in response to oxidative stress. These activated inflammatory mediators induce microglia activation, which leads to the activation of adhesion molecules at the blood-endothelium interface (Stanimirovic et al. 1997). The primary inflammatory mediators are endothelial cells, adhesion molecules, cytokines, and chemokines. The ischemic stimuli cause the detachment of the endothelial cells from the basement membrane, and leads to the activation of the arachidonic acid cascade, promoting platelets and neutrophil activation and BBB permeability (Ishikawa et al. 2004; Yilmaz and Granger 2008). The activated leukocytes near the surface of the endothelium cause no-flow phenomenon and also produce proteases, collagenases to damage blood vessels and brain tissue. Additionally, phospholipase activation within the leukocytes leads to the platelet aggregation and production of various vasoconstrictive agents (Härtl et al. 1996). The earliest type of leukocytes that enters the ischemic region is neutrophils. Neutrophils are proven to exacerbate brain damage via inflammatory mediators after ischemia-reperfusion. The activation of lymphocytes takes place after the activation of neutrophils. The blockage of T-lymphocyte mediated release of cytokines decrease infarct in preclinical stroke models. The adhesion molecules are involved in the establishment of interaction between vascular endothelial cells and immune molecules (leukocytes, lymphocytes, and platelets) and promote immune cell infiltration into brain parenchyma (Q. Wang, Tang, and Yenari 2007). Adhesion molecules mainly comprised of three classes: selectins (P-selectin, E-selectin, and L-selectin), immunoglobulin superfamily (ICAM-I & 2, VCAM-I), and integrins (CD11a-c). Targeting these adhesion molecules prevents immune cell infiltration into ischemic penumbra and reduces neuronal cell injury (Yilmaz and Granger 2008). Cytokine expression is upregulated in the brains of human and preclinical stroke models after ischemia. Cytokines are mainly expressed by immune cells and brain cells like neurons and microglia. The most extensively studied cytokines in stroke models are interleukin-1 (IL-1α and β), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β). Chemokines and their receptors implicated in leukocyte migration and extravasation. Chemokines are primarily expressed by injured neurons, endothelial cells, circulating immune cells, astrocytes, and microglia (Q. Wang et al. 2007). Several transcription factors like nuclear factor-kappaB (NF-kB) and MAP kinases are upregulated following ischemia and promote inflammation. MAPK family proteins are mainly involved in the upregulation of pro-inflammatory mediators following ischemia, and inhibition of the MAPK pathway is shown to reduce infarct and neurological deficit in preclinical stroke models (Irving and Bamford 2002).

Apoptosis:

A few minutes after ischemic stroke, the core region of the brain tissue is fatally damaged and subsequently experiences necrotic cell death. The necrotic core is encompassed by a zone of reduced blood flow and metabolically active tissue known as the ischemic penumbra. Apoptosis is the programmed cell death mechanism implicated in the pathogenesis of acute as well as chronic neurodegenerative disorders. Apoptosis is the dominant cell death mechanism that contributes significantly to neuronal cell death in the penumbra (Sairanen et al. 2006). Necrosis and autophagy cell death modes also occur in the ischemic penumbra. Apoptosis results in the formation of DNA fragments of about 180bp, cellular protein degradation, apoptotic body formation, expression of ligands for receptors of the phagocytic cell, and finally phagocytosis by microglia and astrocytes without eliciting any inflammatory response. The apoptotic signaling cascade is mainly mediated by various killer proteins, which are summarised below. Caspases are evolutionarily conserved cysteine aspartic acid proteases responsible for the apoptotic mode of programmed cell death in mammals. Caspases are mainly subdivided into two broad groups: Interleukin-1 converting enzyme family (caspase-1, 4, 5, 11, 12, and 14), which involves the induction of cytokine-mediated inflammation. The other group are - caspases involved in apoptosis, which are further sub-grouped into initiator caspases (caspase-8, caspase-9, and caspase-10) and effector caspases (caspase-3 and caspase-7). The activation of caspases takes place by two significant pathways, including the intrinsic pathway and extrinsic pathway, which are mediated by the involvement of various signaling molecules and cell surface death receptors (Love 2003). The extrinsic pathway induces apoptotic response via TNF receptor superfamily, including TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand and Fas-associated death domain (FADD). Ligand-induced activation of these receptors results in the activation of pro-caspase-8. The activated caspase-8 works as initiator and helps the activation of terminal caspases such as caspase-3 and neuronal cell loss (Benchoua et al. 2001; Shuaib et al. 2007). The intrinsic pathway of an apoptotic signaling cascade is induced by the activation of Bcl-2 family proteins, which are subdivided into three groups including group-1(anti-apoptotic proteins-Bcl-2, Bcl-xL, Bcl-W), group-2 (pro-apoptotic proteins- Bak, Bax, Bok), and group-3 (pro-apoptotic Proteins-Bid, Bad, Bim, PUMA). The anti-apoptotic function of group-1 proteins may be mediated by sequestration of activated Bax, Bad, and other pro-apoptotic proteins. Further, the intrinsic pathway is initiated by stress-induced disturbances in the mitochondrial membrane. The stress stimuli cause translocation of pro-apoptotic Bcl-2 family members (Bax, Bad) from cytosol to mitochondrial membrane and promote the release of Cyt-c. The cytosolic Cyt-c promotes oligomerization of apoptosis activating factor-1 (Apaf-1), which helps the activation of pro-caspase-9 and forms the apoptosome complex (Cyt-c-caspase-9-Apaf-

1), which activates pro-caspase-3, the terminal mediator of apoptosis (Adams and Cory 2001; Kaufmann and Hengartner 2001; Wang et al. 2001). Both the intrinsic and extrinsic apoptotic pathways can share various common molecular mediators like Bcl2-related protein (BID), which is cleaved by caspase-8 to produce an active form tBID. The activated BID can translocate from the cytosol to mitochondria and induces Cyt-c mediated intrinsic pathway. N-myristoylation facilitates the translocation of tBID to mitochondria at a site that was made available by caspase-8 cleavage (Krajewska et al. 2004; Yin et al. 2002; Zha et al. 2000). Recent findings suggest that cerebral ischemia contributes to the activation of caspase-mediated neuronal cell death. The upregulation and activation of caspases-3 are responsible for neuronal cell loss in experimental stroke models and humans (Sairanen et al. 2009).

p53:

The transcription factor p53 regulates a myriad of cellular processes, including cell-cycle arrest, apoptosis, DNA repair, and metabolism in response to cellular stress (Guo et al. 2015a), (Vousden and Lu 2002a). The expression of p53 is rapidly upregulated in the brain after ischemia and contributes to a significant proportion of neuronal apoptosis. The inhibition of p53 is shown to attenuate neuronal cell loss against ischemia in various experimental animal models (Crumrine, Thomas, and Morgan 1994), (Liu et al. 2019). P53 induced neuronal apoptosis occurs through either a transcription-dependent manner or an independent manner. A transcription-dependent mechanism is an essential tool by which p53 promotes neuronal cell apoptosis by inducing the expression of different target genes like p21, PUMA, and Bax. PUMA is directly activated by p53 and translocate to mitochondria and interacts with Bcl-2, Bcl-xL, and Bax, and promotes Cyt-c release from mitochondria and activates caspase-9 and caspase-3 (Cregan et al. 2004). P21 is also a target of p53 and contributes to the regulation of apoptotic cell death (Van Lookeren Campagne and Gill 1998). Recent evidence supports that p53 directly translocates from cytosol to mitochondria in response to stress stimuli and forms an inhibitory complex with anti-apoptotic Bcl-xL and Bcl-2 and promotes caspase-3 induced apoptotic brain cell death via Cyt-c release from mitochondria (Erster et al. 2004).

MAP Kinase pathway:

The mitogen-activated protein kinase (MAPK) family is characterized as a serine-threonine protein kinase family mainly composed of three members including C-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein kinase p38). MAP kinases play a vital role in stress-mediated signal transduction in mammalian cells. On activation by phosphorylation on both Thr & Ser residues, these kinases phosphorylate various transcription factors and enzymes that involve cellular stress, differentiation, and development. The JNK and p38 are known as stress-responsive MAP kinases, mainly activated by cellular stress like inflammation, apoptosis, and oxidative stress. On the other hand, ERK is activated in response to growth factors and oxidative stress. ERK controls multiple functions, including cell growth, differentiation, and apoptosis. The stress stimuli that enable JNK can also induce p38 activation. The mediators of the p38 and JNK pathway can undergo crosstalk at several levels (Sugino et al. 2000). The mammalian cells express three JNK isoforms include JNK1, JNK2, and JNK3 (Shackelford and Yeh 2006). The first two are ubiquitous in all tissues, and the third is abundantly expressed in the brain and heart (Vlahopoulos and Zoumpourlis 2004). JNK activation plays a critical role in apoptosis induction (Liou et al. 2003). The activated JNK phosphorylates Ser-residue on various pro-apoptotic factors to regulate their transcriptional and post-translational activation. The activation of JNK permits the phosphorylation of C-Jun component of the transcription factor complex AP1, thereby triggers the activation of various apoptotic mediators. JNK also directly involves in the activation of pro-apoptotic p53 and inhibits the anti-apoptotic function of Bcl-2 and Bcl-xL. Recent findings show that the translocation of Bax and Bim to mitochondria and the release of Cyt-c and caspase-3 is JNK dependent (Gao et al. 2005a), (Okuno et al. 2004). The JNK pathway also involves the activation of the inflammatory pathway by targeting various pro-inflammatory mediators, including TNF-a, IL-8 (Zheng et al. 2020). Further JNK can directly inhibit Sirtuin-1 functional activity by phosphorylation, thereby promoting inflammation and apoptosis (Gao et al. 2011). The JNK signaling pathway is upregulated in the brain after cerebral ischemia, and inhibition of the JNK pathway is shown to have functional protection against ischemia in various preclinical stroke models (Guan et al. 2006). Tyrosine kinases and G-protein coupled receptors initiate the activation of ERK through small GTP-binding proteins like Ras. The activated Ras leads to the phosphorylation of Raf and MEK. The activated MEK phosphorylates ERK and promotes the activation of various membrane proteins and transcription factors (Cheung and Slack 2004). Even though ERK is involved in neurite outgrowth and synaptic plasticity, ERK promotes apoptotic cell death in several paradigms (Xu et al. 2003), (Namura et al. 2001). Recent reports suggest that ERK activated during glutamate-induced excitotoxicity and peroxynitrite induced apoptosis (Oh-Hashi, Maruyama, and Isobe 2001; Stanciu et al. 2000). ERK activation is also reported in other neurodegenerative disorders like Alzheimer's, where ERK activation is observed in degenerating neurons (Pei et al. 2002). The expression of the ERK signaling pathway is increased in the brain after cerebral ischemia, and inhibition of the MEK, the upstream activator of ERK pathway is shown to have neuroprotection against ischemia in experimental stroke models

(Alessandrini et al. 1999). The role of ERK in cell death probably depends on the cell type as well as on the intensity of the stress stimuli.

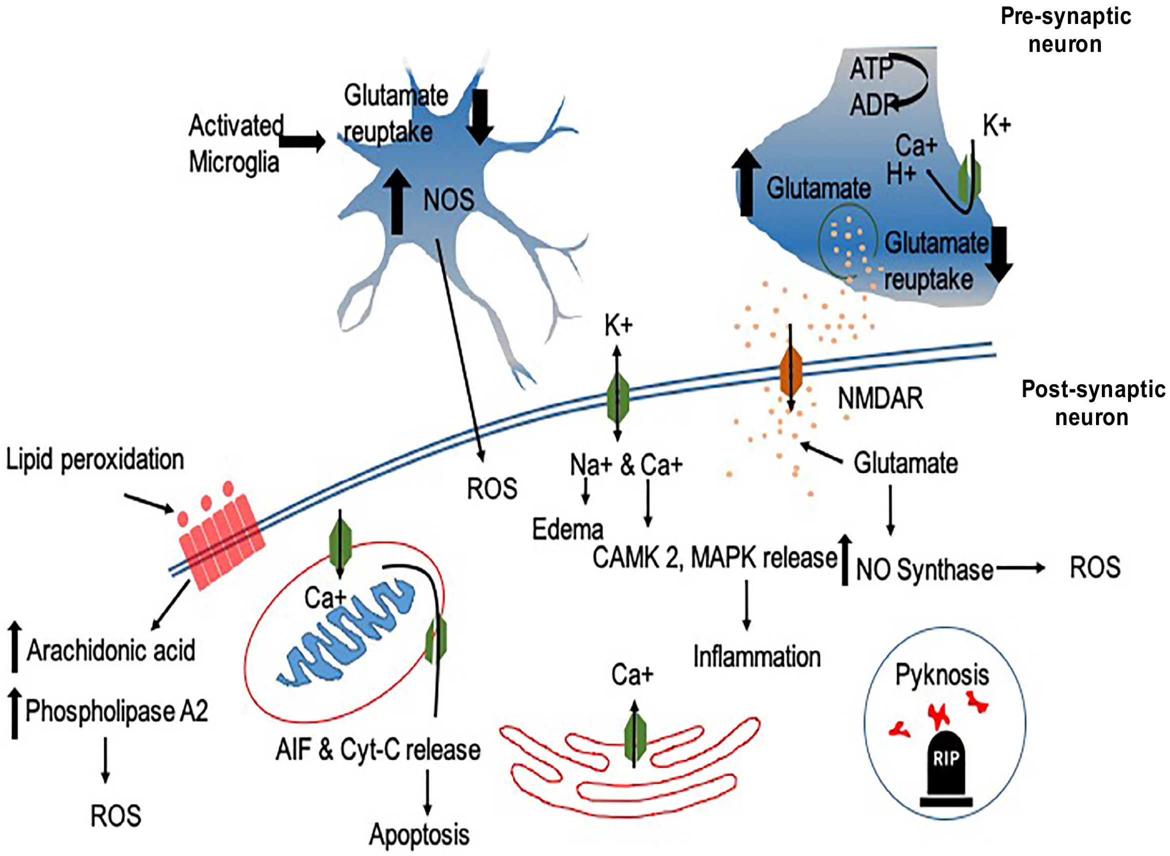
Autophagy:

Autophagy is a conserved degradation process, where long-lived cytoplasmic proteins and damaged organelles are engulfed by a double membrane autophagosome and flux with lysosomes for degradation. Typically, autophagy activation takes place during cellular stress and nutrient deprivation and represents a central role in cellular homeostasis (Cai et al. 2019). A complex network of multiple proteins mediates autophagy induction in a non-linear fashion. An extensive compendium of autophagy related genes (Atg) implicated in autophagosome formation and maturation. The autophagy pathway is mainly constituted of four steps: initiation/nucleation, autophagosome formation, trafficking/maturation, and recycling/release of macromolecules. The dephosphorylation of the mammalian target of rapamycin (mTOR)/activation of Beclin-1/phosphatidylinositol-3 kinase (PI3K) complex initiates autophagy mechanism in response to cellular and nutrient stress. This complex is essential for the extension of the isolation membrane (IM). After nucleation, autophagosome formation occurs via two ubiquitin-like conjugation systems, including Atg12-Atg-5 complex and microtubule-associated protein 1a/1b light chain-3 (Lc-3a/b)/ Atg8. The first conjugation system activation is initiated by Atg-7, which activates ubiquitin-like protein Atg-12. The activated Atg-12 is transferred to Atg-10 and conjugates to Atg-5. The Atg-12-Atg-5 complex eventually interacts with Atg-16L1 forming the Atg-12-Atg-5-Atg-16L1 complex. This complex is necessary for the elongation of the IM for autophagosome formation. The second conjugation system Lc-3a/b is processed at the c-terminus by Atg-4 to form cytosolic Lc-3I, which is converted to Lc-3II when conjugated to phosphatidylethanolamine (PE). Lc3-II binds with the inner and outer surface of the autophagosome membrane. This mechanism is activated by Atg-7. After binding with autophagosome membrane, Lc-3II is either degraded after lysosome fusion or removed via de-conjugation. Hence Lc-3II is considered a critical indicator of the cytosolic autophagosome pool (Mizushima, Ohsumi, and Yoshimori 2002). The basal autophagy is neuroprotective, while excessive/dysregulated autophagy promotes cell death in various chronic and acute neurodegenerative disorders, including Alzheimer’s, Niemann-pick, Huntington, and cerebral ischemia (Nixon and Yang 2011), (Martinez-Vicente et al. 2010), (Elrick et al. 2012), (Jiang et al. 2017). Autophagy can share common molecular mediators with other cellular pathways that promote cell survival and cell death. The Bcl-2 and Bcl-xL are well established anti-apoptotic proteins that can bind to Bax and limit its mitochondrial translocation and succeeding release of mitochondrial Cyt-c, thereby activating caspase-3. Bcl-2/Bcl-xL can bind to Beclin-1 and prevents autophagy initiation. Once the Bcl-2/Bcl-xL-Beclin-1 complex is disrupted, Beclin-1 will induce an autophagy mechanism (Boya et al. 2005; Oberstein, Jeffrey, and Shi 2007; Pattingre et al. 2005; Qi et al. 2015). The JNK can phosphorylate Bcl-2 at T69, S70, and S87A sites and prevents Bcl-2 interaction with Beclin-1 and promotes autophagy induction (Wei et al. 2008). Additionally, constitutive activation of JNK promotes Bcl-2 phosphorylation and increases apoptotic cell death (X.-T. Wang et al. 2007). More importantly, Beclin-1-Lc-3 interaction inhibits the activation of pro-apoptotic Bim (Luo et al. 2012). Disabled-2 promotes the phosphorylation of Beclin-1 via protein casein kinase 2 and leads to the disruption of Beclin-1-vps34 complex and prevents autophagy (Jiang et al. 2016). The scaffold protein β-Arrestin interacts with Beclin-1-vps34 complex in neurons, and the interaction between Beclin-1-vps34 complex was disturbed by the removal of b-Arrestin, thereby autophagosome formation and also promotes caspase-3 mediated apoptosis after cerebral ischemia (Pei Wang et al. 2014).

Sirtuin-1:

Sirtuins are well-known class-III histone deacetylases. They remove the acetyl group from lysine by hydrolysis on histones and non-histones. Sirtuins consume one NAD moiety for every acetyl group they removed. There are seven human homologs of Sirtuins (Sirt1-Sirt7). Among them, mammalian Sirt2 ortholog Sirt1 is well-characterized Sirtuin. Sirt1 mediates caloric restriction induced life span extension and aging in lower eukaryotes (Li et al. 2008). Sirt1 regulates a myriad of metabolic and stress-responsive factors. Sirt1 is expressed at a higher level in neurons and the adult brain. Sirt1 is proven to be protective against age-induced chronic and acute neurodegenerative disorders (Gan and Mucke 2008). Sirt1 mediated protection against Alzheimer's mainly comes from the deacetylation of tau and retinoic acid receptor-beta (RAR-b), thereby prevents amyloid-b (Ab) plaques formation. In Parkinson's disease, Sirt1 prevents aggregation of a-synuclein by deacetylation of heat shock factor 1 (HSF1) and activation of molecular chaperone signaling. In Huntington's disease, Sirt1 activates brain-derived neurotrophic factor (BDNF) and supports neuronal survival (Donmez 2012a). Sirt1 is reported to be neuroprotective during ischemic preconditioning and post-stroke resveratrol treatment. Sirt1 activation is reported to reduce infarct and neuronal injury in various experimental stroke models by targeting inflammatory and apoptotic mediators (Yan et al. 2013b), (Della-Morte et al. 2009). Sirt1 can directly deacetylate various autophagy inducers like FOXO, p53, and HIF-1, thereby control autophagy induction. The tumor-suppressor protein p53 promotes the activation of DNA damage-regulated autophagy modulator (DRAM), and glycolysis and apoptosis regulator (TIGAR), which can stimulate autophagy induction (Cheung, Ludwig, and Vousden 2012; Crighton et al. 2006). The hypoxia-inducible factor-1 (HIF-1) is a significant regulator of hypoxic stress. The increased expression of HIF-1 promotes transcription of BCL2/adenovirus E1B interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L), thereby supports the autophagy mechanism (Mahalingaiah and Singh 2014). The transcription factor FOXO3 can directly enhance the LC3 and BNIP3 transcription to aid autophagy induction. The NAD-dependent deacetylase Sirtuin-1 modulates autophagy by directly interacting with essential components of autophagy, including Atg-5, Atg-7, and Lc-3 (Ren et al. 2019). The inhibition of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in NAD biosynthesis, impairs autophagy mechanism in Sirt1 dependent manner in mouse MCAO model (Wang et al. 2012).

Acute neurochemical changes after ischemia:



The schematic representation of the acute pathophysiological changes in the brain during cerebral ischemia.

Chapter-1

Differential role of Sirt1/MAPK pathway during cerebral ischemia in rat and human.

1 Introduction:

Ischemic stroke is the most common subtype of stroke (Shiber, Fontane, and Adewale 2010b). Stroke can affect individuals of any age. Aged individuals experience the highest incidence of ischemic stroke and poor functional recovery (Feigin, Norrving, and Mensah 2017). The majority of the stroke studies are on young experimental animal models. The molecular mechanisms of injury and risk factors for stroke differ between rodents and humans (Ritzel et al. 2018). The studies with rodents and human subjects could help to avoid false conclusions and gain a better understanding of stroke pathophysiology. Apoptosis is the programmed cell death; the presence of apoptotic-like cells is a hallmark of the peri-infarct region. Caspase-3 is an critical mediator of apoptotic brain cell death in the ischemic hypo perfused peri-infarct area (Sairanen et al. 2006).

Ischemic reperfusion activates various stress-responsive protein kinases. Mitogen-activated protein Kinase (MAPK) are a family of serine/threonine kinases that play a critical role in the regulation of neuronal survival and apoptosis (Shackelford and Yeh 2006), (T.-C. Wu et al. 2000). c-JUN N-terminal kinase (Jnk1/2), an important subgroup of the MAPK family, is activated by I/R injury and is shown to be implicated in apoptotic neuronal cell death (Okuno et al. 2004). On the other hand, growth factors are linked to the activation of extra cellular signal regulated kinase (Erk1/2), a subfamily of MAPK pathway and Irs/Akt pathway (Zhou et al. n.d.). Even though Erk1/2 is activated in response to growth stimuli and contributes to neuronal cell proliferation and differentiation (Xu et al. 2003), it also acts as a key regulator of apoptotic neuronal death (Cheung and Slack 2004). Under the influence of growth factors Irs-1activates PI3K and recruits Akt thereby promoting neuronal survival. Evidences have shown that Akt phosphorylation inactivates various pro-apoptotic factors and decrease neuronal cell death after cerebral ischemia and activation of Akt is also shown to be negatively regulate Mek1/2/Erk1/2/MAPK pathway (Noshita et al. 2001), (Yoshimoto et al. 2002).

Sirt1 is an evolutionarily conserved class-III NAD-dependent deacetylases, which plays a significant role in longevity and cell survival by deacetylation of various stress-responsive transcription factors involved in inflammation and apoptosis (S Imai et al. 2000), (Yeung et al. 2004a), (Vaziri et al. 2001a). Several lines of evidence have demonstrated that Sirt1 synergistically interacts with the MAP Kinase pathway and Sirt1 upregulation affects MAPK pathway and inhibits apoptotic cell death in experimental models (Wang et al. 2009). Sirt1 directly affects Akt activity by deacetylation and promotes neuronal differentiation (Li et al. 2013). However, the definite role of Sirt1/MAPK/Akt pathway during ischemia in aged models and humans has not been explored.

In this connection, our present study investigated the role of Sirt1 in the brains of rat and human and explored its involvement in the regulation of Jnk1/2/Erk1/2/MAPK/Akt pathway mediated apoptosis, which may help in the development of effective therapeutic strategies.

2 Materials & Methods:

2.1 Transient Middle Cerebral Artery Occlusion (MCAO):

All the animal trials were conducted following the guidelines of the Institutional and National Animal Ethical Committees (IAEC). Young (3-4 months) and aged (18-20 months) male Sprague-Dawley (SD) rats were obtained from NCLAS (The National Centre for Laboratory Animal Sciences), India. Animals moved to rodent house facilities were under controlled conditions with unrestricted access to water and food. The rats were randomly distributed into six groups: (i) Sham group, (ii) Control group (DMSO + PBS), (iii) MCAO group, (iv) MCAO + EX-527 group (Intracerebroventricular (ICV) route; 30 µg/kg; before 30 min of MCAO), (v) MCAO + Resveratrol group (Intraperitoneal (IP) route; 20mg/kg; after 30min of MCAO), (vi) MCAO + SP600125 group (ICV route; 30 µg /kg; before 30min of MCAO). All the drugs were purchased from Sigma (USA). All attempts were made to reduce the animal number to be used to decrease animal suffering.

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO), which was adopted as per the protocol of Longa et al, (Longa et al. 1989a). Briefly, rats were anaesthetized with Ketamine (60mg/Kg) and Xylazine (10mg/Kg) administration via IP route. The middle neck incision was made to locate arteries mainly the left common carotid artery (CCA), internal carotid artery (ICA), and the external carotid artery (ECA). Microvascular clips were temporarily placed on CCA and ICA, while ECA was distally ligated, a 3-0 nylon monofilament was inserted from ECA and gently introduced into ICA to occlude the MCA origin. The occlusion of MCA origin was perceived through the resistance created. For the sham surgery, nylon filament was advanced into CCA, but the origin of MCA was not occluded. After completion of 60 min of occlusion, the nylon monofilament was removed smoothly to restore blood flow, and rats were left to recover.

2.2 Evaluation of neurological deficit:

Neurological deficit was evaluated for infarct intensity by Bederson score (Bederson et al. 1986). Rats with no observed behavioral deficiency were considered as grade 0, rats which failed to extend contralateral forelimb to the infarct side were considered as grade 1, rats with diminished resistance to lateral push with occasional circling towards ipsilateral side were considered as grade 2, rats falling to the contralateral side were considered as grade 3, and rats that died due to severe lesion was considered as grade 4. The experimental rats with neurological scores below grade 3 were excluded from experimental groups.

2.3 Infarct volume measurement:

Brains coronal slices with 2 mm thickness were stained with 2% TTC (2,3,5- triphenyl tetrazolium chloride) at 37° C for 30 min. After staining brain slices were fixed in 4% paraformaldehyde (PFA). Sections were filmed and analyzed with ImageJ software. Infarct areas from all segments were added to derive the total infarct, which was multiplied by the thickness of the brain section to get total infarct volume. Correction for edema of infarct was performed as described previously (Lin et al. 1993).

2.4 Immunoblotting:

Rats were executed with the excess of pentobarbital at 24 h following MCAO. The ipsilateral brains were extracted and homogenized in RIPA buffer (150mM NaCl,2mM EDTA,50mM Tris-HCl(pH-7.4), 10%Glycerol, 1%NP40, & 0.5%Sodium deoxycholate). The brain tissue was homogenized at 10,000 rpm for 20 min, and the total amount of proteins in the supernatant was estimated by using Bradford reagent. Western blots probed an equal amount of total protein sample (75g). Total protein extract was resolved on 8% and 10% SDS-PAGE gels and the resolved proteins were transferred on to nitrocellulose membrane (NCM). The NCM was covered with 5% non-fat dry milk (HIMEDIA, India) in Tris-buffered saline containing Tween-20 (TBS-T) for 1 h 30 min at room temperature (RT). The NCMs were covered with 1° Ab overnight at 4° C (1:1000) against Sirt1, phospho & total-Jnk1/2, phospho & total-Erk1/2, phospho & total-Mek1/2, phospho & total-Akt, beta-tubulin and phospho-Irs-1 (ser307). Membranes were washed with TBS-T and covered with HRP conjugated 2° Ab for 1 h 30 min at RT (1:3000; rabbit & mouse). The chemiluminescence was captured using photographic film and the ECL detection system. All the primary and secondary antibodies were from Cell Signaling Technology (CST, USA) except p-Irs, which was purchased from Merck, USA.

2.5 Immunohistochemistry:

Brains at 24 h after reperfusion were fixed by trans cardiac perfusion with PBS and 4% PFA. The paraffin-embedded brains were sectioned into 5µm thick slices utilizing a microtome. Brain sections were immunostained against Sirt1, p-Jnk1/2, and caspase-3. Brain sections were dewaxed in three xylene washes each for 5 min, then hydrated in a decreasing gradient of alcohol for 5 min each. Peroxidase was inhibited by quenching sections with 3% hydrogen peroxide in methanol. After peroxidase inhibition, sections were covered in citrate buffer (pH-6.0) for heat-induced antigen retrieval. After cooling, sections were covered with 0.25% BSA for 30 min to minimize non-specific binding. Sections were treated with the 1° Ab (1:100 dilution) in blocking solution for 2 h 30 min at 37° C, followed by HRP-labelled 2° Ab incubation for 45 min at 37° C (provided with the kit). Staining was envisioned by covering the sections in 3'-3' diaminobenzidine buffer(DAB) for 5 min, counterstained by Haematoxylin. After dehydration, sections were mounted, and photographs were taken under the microscope with 400 X magnification.

2.6 Haematoxylin & Eosin (H & E) staining:

Brain sections were dewaxed and hydrated in a decreased gradient of alcohol, followed by staining with Haematoxylin and Eosin. Sections were dehydrated, cleared in xylene and mounted. Images were observed under the light microscope for the presence of morphological changes under 400 X magnification.

2.7 Immunofluorescence:

Triple immunofluorescence labeling was performed to probe for Sirt1, DAPI (CST, US), and Fluoro Jade-C (FJ-C), (Millipore, US), which stains degenerating neurons. Sections from both experimental rat and humans were deparaffinized and rehydrated through 100-70% graded ethanol in water. Sections were covered in citrate buffer and boiled for three times, each for 5 min with 1 min interval in a microwave. Sections were blocked by 5% goat serum for 60 min at 37° C and Incubated in anti Sirt1 primary antibody for overnight at 4°C. After being washed with PBS, brain sections were covered with Alexa Fluor – 555 conjugated goat anti-mouse IgG (CST, US) for 60 min at room temperature. After PBS washing, sections were covered with 0.06% KMno4 for 10 min, followed by Fluoro Jade-C incubation for 30 min at 37° C. Slides were mounted with antifade DAPI and Images were digitalized under the laser scanning confocal microscope (Carl-Zeiss, Germany).

2.8 Post-mortem human brain tissue:

Autopsied ischemic human brain tissues were obtained from “Human Brain Bank, Department of Neuropathology, National Institute of Mental Health & Neurosciences (NIMHANS); Bangalore, India.” All study procedures on human subjects were approved by the Institutional Ethical Committee (IEC), University of Hyderabad, India. The informed consent to commence autopsy was obtained from family members of the patient. Healthy brain tissue without any neurological abnormality was used as control. Patient details including age, sex, and region of infarct are given in the Table-1. The brain tissue was homogenized in RIPA buffer using the same method stated in the immunoblotting section. Total protein extract was separated on 10% SDS-PAGE gels and antibody used against Sirt1, phospho & total Jnk1/2, phospho & total Erk1/2, phospho & total Akt, and beta-actin. The chemiluminescence was visualized using the ECL detection system (Bio-Rad, USA). Brain tissues were embedded in paraffin following formalin fixation. Formalin-fixed brains were sectioned into 5μm thick slices and stained with Haematoxylin & Eosin to assess morphological changes. Immunohistochemistry was performed against Sirt1, p-Jnk1/2, & caspase-3 using the method as mentioned in the immunohistochemistry section.

3 Results:

3.1 The decrease in Sirt1 expression & phosphorylation of Jnk1/2/Erk1/2/MAP Kinases in brains of experimental aged rat following MCAO:

To examine the change in expression of Sirt1 in the ischemic rat model, we quantified Sirt1 protein expression using western blot analysis following 24 h of ischemia. A significant decrease was observed in Sirt1 protein expression in the brains of young and aged experimental rat compared to control (Sirt1-$p<0.001$, Figure. 2 A). Further, we investigated the functional relation between MAP Kinases and Sirt1 in the brains of aged experimental rat. The phosphorylation status of MAP Kinase family proteins including Jnk1/2, Mek1/2, and Erk1/2 was determined by immunoblotting and found a substantial decrease in Jnk1/2/Erk1/2/MAP Kinase phosphorylation following MCAO compared to sham control (p-Jnk1/2-$p<0.001$; p-Mek1/2- $p<0.001$; p-Erk1/2-$p<0.02$, Figure. 2 B-D).

On the contrary, in young ischemic rats, there is a significant increase in the phosphorylation status of stress-induced Jnk1/2/Erk1/2/MAP Kinases compared to control (p-Jnk1/2-$p<0.007$; p-Mek1/2-$p<0.001$; p-Erk1/2-$p<0.001$, Figure. 2 B-D).

3.2 Subcellular localization of Sirt1, p-Jnk1/2, & caspase-3 following MCAO in aged rats:

To determine the cellular distribution of Sirt1, p-Jnk1/2, & caspase-3 in rats following ischemia, we immunostained paraffin-embedded brain sections from control and 24 h after reperfusion from both the age groups (young & aged rats). Routine H&E staining showed the pyramidal neuronal loss (black arrow – pyknotic neuron) in both cortex and hippocampus following MCAO (Figure. 1 B & G). TTC staining was performed to assess the infarct size (Figure. 1 K & L). Sirt1 immunoreactivity is mainly observed in the nucleus of cortex and hippocampus neurons in control brains. Post-ischemic Sirt1 immunoreactivity was decreased in nucleus and more predominant in cytoplasm and cell processes of neurons compared to control in both young and aged experimental rats (Figure. 6 A & B, Figure. 7 A & B).

We observed p-Jnk1/2 immunoreactivity in both cytoplasm and nucleus of neurons in aged controls. p-jnk1/2 immunoreactivity was decreased in aged rats following MCAO. On the contrary, in young experimental rats, we found an increased immunoreactivity of p-Jnk1/2 following MCAO (Figure. 7 G), (Figure. 6 F & G). Moreover, we observed caspase-3 immunoreactivity in neuronal cytoplasm of control and immunoreactivity of caspase-3 is mainly seen in nucleus following MCAO (Figure. 6 K & L, Figure. 7 K & L).

3.3 Increase in Sirt1 expression & Jnk1/2/Erk1/2/MAPK, & Akt phosphorylation in post-mortem human stroke brain tissue:

To determine the change in expression of Sirt1, and phosphorylation of Jnk1/2/Erk1/2/MAPK, and Akt in human stroke brain, we measured Sirt1, Jnk1/2/Erk1/2/MAPK, and Akt protein expression using western blot analysis. We found that Sirt1 protein expression was increased in the stroke brain compared to control (Figure. 10 E). Furthermore, phosphorylation of Jnk1/2/Erk1/2/MAPK and Akt was also increased in the ischemic brain (Figure. 10 F, G, & H).

The presence of neurodegeneration in stroke brain was showed using H & E staining of paraffin-embedded brain sections (black arrow, Figure. 10 D). The subcellular distribution of Sirt1, p-Jnk1/2, and caspase-3 were studied using immunohistochemistry in post-mortem brain tissues. Sirt1 immunoreactivity is increased in stroke brain, but in control, we observed both nuclear as well as cytoplasmic Sirt1 immunoreactivity (Figure. 10 A). These results were further supported by immunofluorescence (IF) study, which showed an increased number of Sirt1 positive cells co-localised with FJ-C within stroke brain compared to control (Figure. 10 I & J). Moreover, we observed increase in p-Jnk1/2 immunoreactivity in stroke brains. We observed nuclear reactivity of caspase-3 in stroke brains, while in control tissue, caspase-3 reactivity was limited to the cytoplasm (Figure. 10 B & C).

3.4 Sirt1 activation with Resveratrol modulates Jnk1/2/Erk1/2/MAP Kinase pathway following MCAO in aged rats:

To understand the interaction of Sirt1 with Jnk1/2/Erk1/2/MAPK pathway during the neuroprotection, resveratrol treatment was given for the activation of Sirt1 in aged rats. Our results substantiated that activation of Sirt1 increased phosphorylation of Jnk1/2/Erk1/2/MAP Kinase family proteins in the brains of resveratrol-treatment compared to the brains of stroke rat (p-Mek1/2–$p<0.001$, Sirt1-$p<0.004$, Figure. 3 A-D), (Figure. 2 A-D). Furthermore, increased immunoreactive neuron nucleus and cytoplasm were seen in cortex and hippocampus against Sirt1, and p-Jnk1/2 on resveratrol treatment compared with the MCAO group (Figure. 6 D & I). To confirm that the neuroprotective effect of resveratrol in aged experimental rats is associated with downregulation of apoptosis, we checked for caspase-3 immunoreactivity and found that there was no decrease in caspase-3 immunoreactivity (Figure. 6 N), a reduction in infarct was observed, which was not significant enough compared to the MCAO group (Figure. 1 I & L). These results were further supported by fluorescence immunohistochemistry, which shown that Fluoro Jade-C predominantly co-localized with Sirt1 in the resveratrol-treated group (Figure. 8 G & H).

In contrast, resveratrol conferred neuroprotection in young rats decreased caspase-3 and neuronal cell death (Figure-6 N & Figure-1 D), (Figure. 9 G & H) by up-regulation of Sirt1 and down-regulation of Jnk1/2/Erk1/2/MAP Kinases (p-Jnk1/2–$p<0.001$; p-Erk1/2–$p<0.001$, Figure. 3 A-D).

3.5 Sirt1 inhibition with EX-527 decreases Jnk1/2/Erk1/2/MAPK phosphorylation following MCAO in aged rats:

The relation between Sirt1 and Jnk1/2/Erk1/2/MAP Kinases is established by the inhibition of Sirt1 with Ex-527 that promotes neurodegeneration in aged rats by decreasing MAP Kinase phosphorylation. Here the Pharmacological inhibition of Sirt1 significantly downregulated the phosphorylation of Jnk1/2/Erk1/2/MAP Kinases in the brains of EX-527 treatment compared to the control (p-Jnk1/2–$p<0.001$; p-Erk1/2–$p<0.001$; p-Mek1/2–$p<0.001$, Figure. 4 B-D). The immunoreactivity of Sirt1 is found to be decreased in the presence of EX-527 compared to I/R group (Figure. 6 C & B).

Moreover, the immunoreactivity of p-Jnk1/2 was decreased in the brains of EX-527 treated aged rat. The neuronal injury was confirmed by caspase-3 immunoreactivity (Figure. 6 H, G & M), and increased infarct and neurodegeneration compared to control, (Figure. 8 E & F), (Figure. 1 Sirt1, F & L). This downregulation of Jnk1/2/Erk1/2/MAP Kinases by Sirt1 inhibition proved that Sirt1 conferred neuroprotection by associating with Jnk1/2/Erk1/2/MAP Kinases in the aged focal ischemic rat.

On the contrary, Sirt1inhibition with EX-527 in the brains of young experimental rat significantly increased neuronal cell death (Figure. 9 E & F) by upregulation of pro-apoptotic caspase-3 (Figure. 7 M), and phosphorylation of stress-responsive Jnk1/2/Erk1/2/MAP Kinases (p-Jnk1/2–$p<0.001$; p-Erk1/2–$p<0.001$; p-Mek1/2–$p<0.001$, Figure. 4 B-D).

3.6 Jnk1/2 inhibition with SP600125 aggravates neuronal damage following MCAO in aged rats:

The synergistic relation between Sirt1 and Jnk1/2/Erk1/2/MAPK and their neuroprotective nature in aged brains during an ischemic injury was confirmed by inhibiting Jnk1/2 activity with SP600125. Here a significant decrease in Mek1/2/Erk1/2 phosphorylation was observed compared to control (p-Mek1/2–$p<0.001$; p-Erk1/2–$p<0.002$, Figure. 5 C, D). The immunoreactivity of p-Jnk1/2 is decreased in the presence of SP600125 compared to MCAO group (Figure. 6 J, G). The expression levels and subcellular localization of Sirt1 were tested and found that there was no substantial change in Sirt1 expression on SP600125 treatment compared to the brains of stroke rat (Figure. 5 A), (Figure. 6 E, B). Additionally, we found increased caspase-3 immunoreactivity on Jnk1/2 inhibition in experimental aged rats compared to control (Figure. 6 O, K). Additionally, immunofluorescence study has shown an increase in the number of Fluoro Jade-C positive cells co-localized with Sirt1 (Figure. 8 I, J). Moreover, Jnk1/2 inhibition increased neuronal death (Figure. 1 J, G) and infarct compared to MCAO group (Figure. 1 L). These results suggested that MAP Kinase- associated neuroprotection in aged rats is mediated by Sirt1.

In contrast, the inhibition of Jnk1/2 activity in young experimental rats during ischemia significantly decreased pro-apoptotic caspase-3 (Figure. 7 O), and neurodegeneration (Figure. 9 I & J) by inhibition of MAP Kinases compared to control (p-Mek1/2–$p<0.001$; Figure. 5 B-D).

3.7 Sirt1 modulates Irs-1/Akt pro-survival pathway following MCAO in aged rats:

The role of Sirt1/Jnk1/2 axis on the phosphorylation status of Irs-1/Akt signaling was established. Here an increase in Irs-1 phosphorylation at ser-307 and decrease in Akt phosphorylation was observed in both young and aged rats following MCAO compared to sham control (p-Irs-1–$p<0.001$; p-Akt–$p<0.001$, Figure. 2 E, F). More importantly, Irs-1 phosphorylation at Ser-307 was increased in aged rats following MCAO even though there was a substantial decrease in Jnk1/2 phosphorylation in brains of aged experimental rat following MCAO.

Further, in the brains of aged rat, Jnk1/2 inhibition with SP600125 decreased Akt phosphorylation but failed to affect the phosphorylation status of Irs-1 at ser-307 compared to control, which was contrary to young rats (p-Akt–$p<0.001$; p-Irs-1–$p<0.001$, Figure. 5 E, F). Moreover, Sirt1 activation on resveratrol treatment decreased Irs-1 phosphorylation at ser-307 and increased Akt phosphorylation in both young and aged rats (p-Akt–$p<0.03$; p-Irs-1–$p<0.001$, Figure. 3 E, F). Sirt1 inhibition with EX-527 increased Irs-1 phosphorylation at ser-307 and decreased Akt phosphorylation and resveratrol treatment abolished the effect of EX-527 in both groups (p-Irs-1–$p<0.001$, Figure. 4 E, F). These results demonstrated that activation of Akt pro-survival pathway is dependent on Sirt1.

Figures:

Figure. 1:

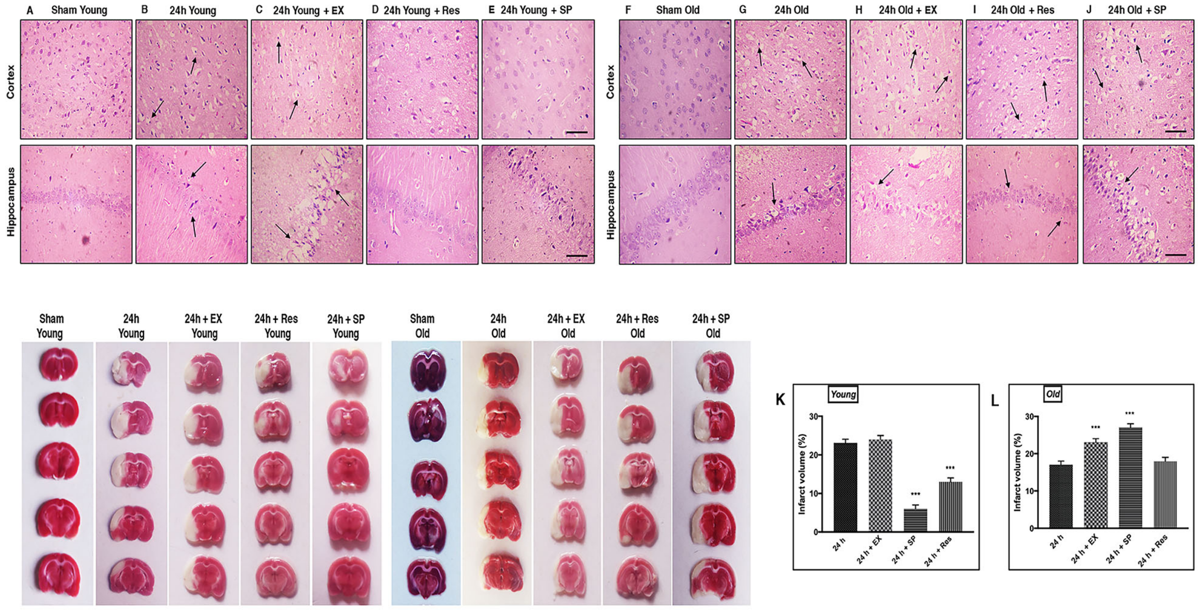


Fig. 1 Assessment of morphological changes following I/R injury. The morphological changes in cortex and hippocampus neurons were assessed using H&E staining (magnification 400X; Scale Bar - 50μm). (A, B & F, G) Represent histological changes after 24 h (pyknotic neuron - black arrow) in young and aged rats compared to respective controls. (C & H) Neuronal loss in both groups after EX-527 treatment. (D & E) Reduced neuronal loss in young rats in the presence of resveratrol and SP600125. (I & J) Neuronal cell death in aged rats, in the presence of SP600125, and resveratrol, where SP600125 and resveratrol failed to show any rescue effect on aged brain. The infarct intensity measured with TTC staining. (K) Resveratrol and SP600125 treatment decreased infarct volume and abolished the effect of EX-527 in the brains of young rat. (L) In the brains of aged experimental rat EX-527 and SP600125 increased infarct and resveratrol failed to reduce infarct. The data are presented as a percentage of tissue loss (n=3). *p<0.05 versus ischemic brain. ***Sham*** - control; ***24 h*** – 24 h MCAO; ***24 h + EX*** – 24 h EX-527; ***24 h + Res*** – 24 h + Resveratrol; ***24 h + SP*** – 24 h + SP600125.

Figure. 2:

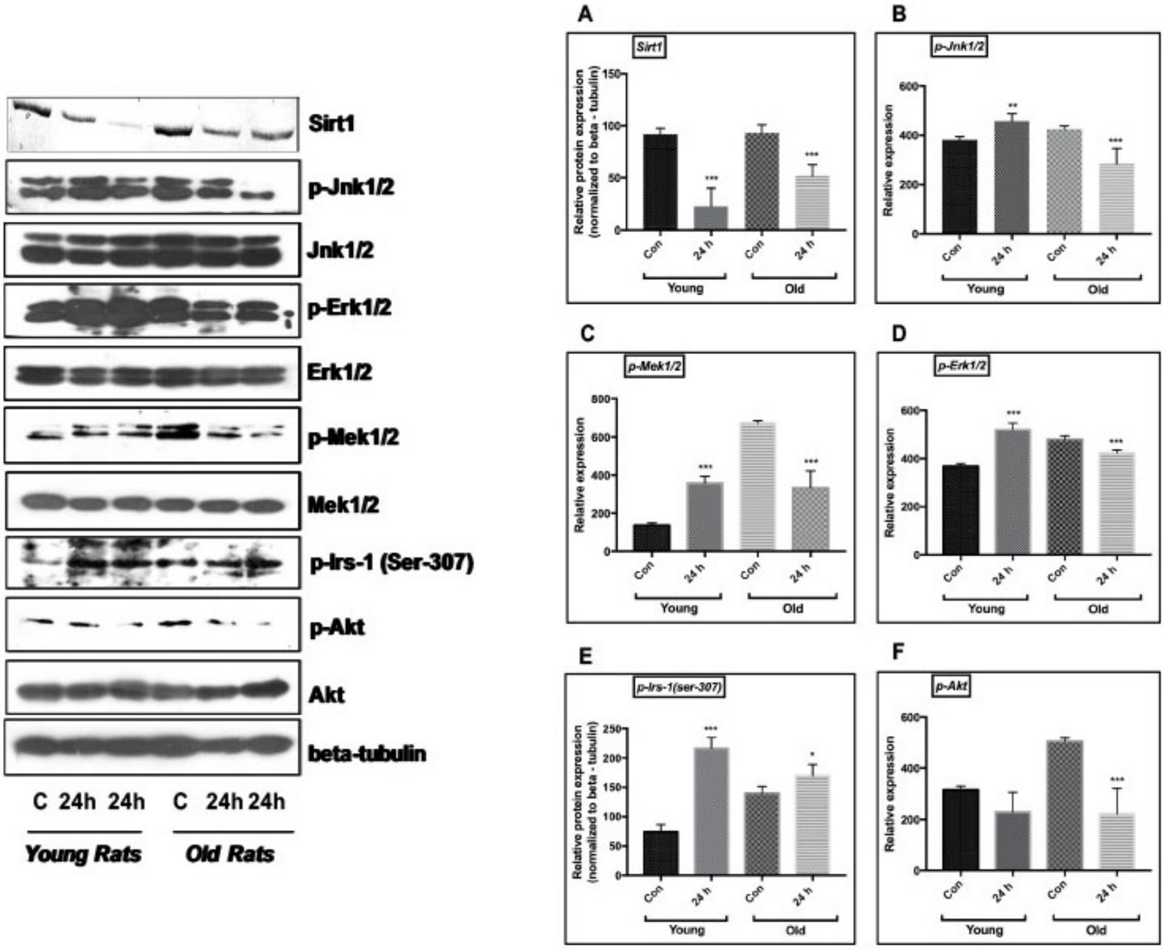


Fig. 2 Effect of ischemia on the expression of Sirt1/MAPK/Akt signalling pathway in brains of aged experimental rat. Control and experimental brains were collected at 24 h after reperfusion. An equal amount of total protein sample (75g) was probed by western blots to analyze the activity of Sirt1 and phosphorylation status of p-Jnk1/2, p-Mek1/2, p-Erk1/2, p-Irs-1, and p-Akt. Respective total proteins and beta-tubulin were served as the loading control (p-Jnk1/2, p-Mek1/2, p-Erk1/2, and p-Akt), while Sirt1 and p-Irs-1 were normalized to beta-tubulin. (**A-F**) Graphs represent the changes in expression of Sirt1/MAPK/Akt pathway. The densitometry values are represented as mean ± SD. $*p<0.05$ versus control brain. ***Con*** - control; ***24 h*** – 24 h MCAO.

Figure. 3:

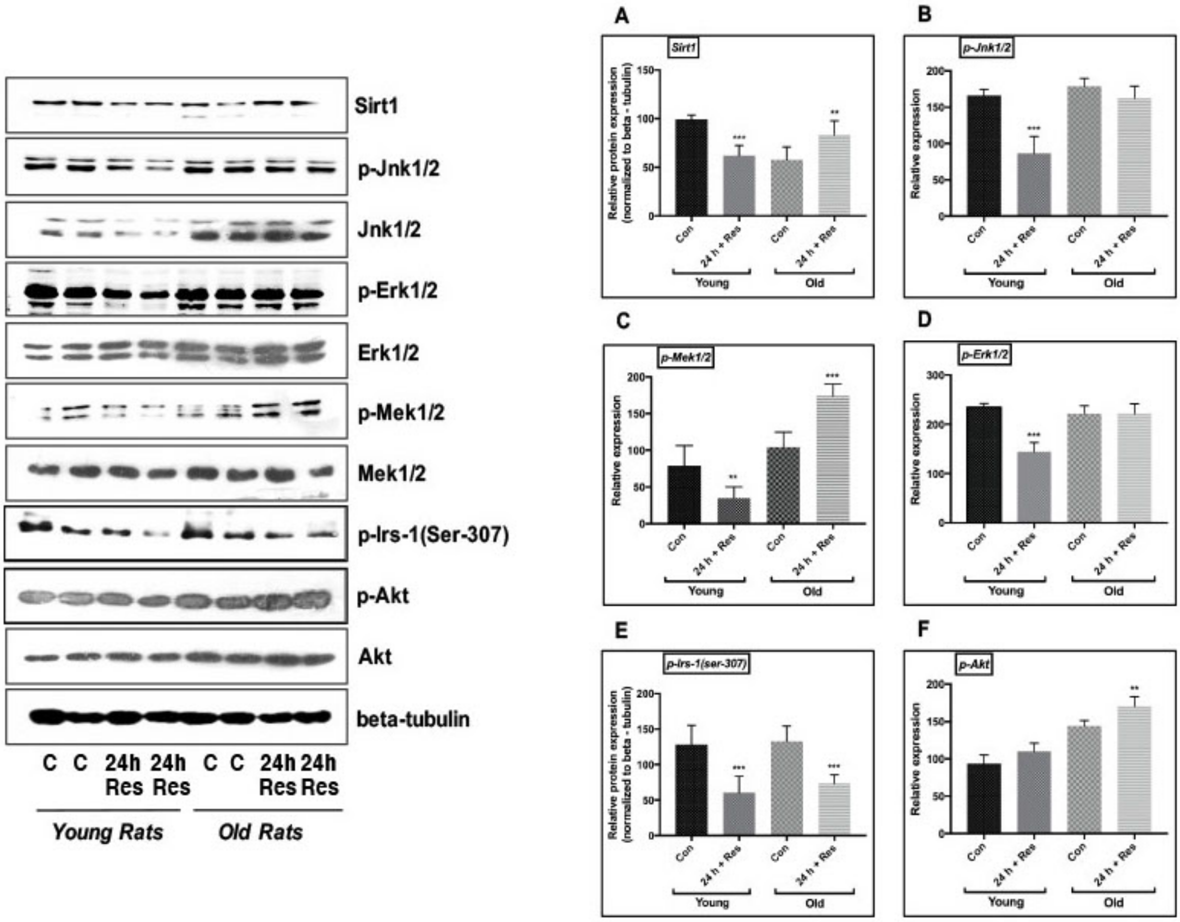


Fig. 3 Resveratrol treatment upregulates Sirt1/MAPK/Akt signalling pathway in aged experimental rats after I/R. Control and experimental brains were collected at 24 h after resveratrol treatment. An equal amount of total protein sample (75g) was probed by western blots to analyze the activity of Sirt1 and phosphorylation status of p-Jnk1/2, p-Mek1/2, p-Erk1/2, p-Irs-1, and p-Akt. Respective total proteins and beta-tubulin were served as the loading control (p-Jnk1/2, p-Mek1/2, p-Erk1/2, and p-Akt), while Sirt1 and p-Irs-1 were normalized to beta-tubulin. (**A-F**) Graphs represent the changes in expression of Sirt1/MAPK/Akt pathway after resveratrol treatment. The densitometry values are represented as mean ± SD. $*p<0.05$ versus control brain. ***Con*** - control; ***24 h Res*** – 24 h MCAO + Resveratrol; ***I/R*** – Ischemia-reperfusion.

Figure. 4:

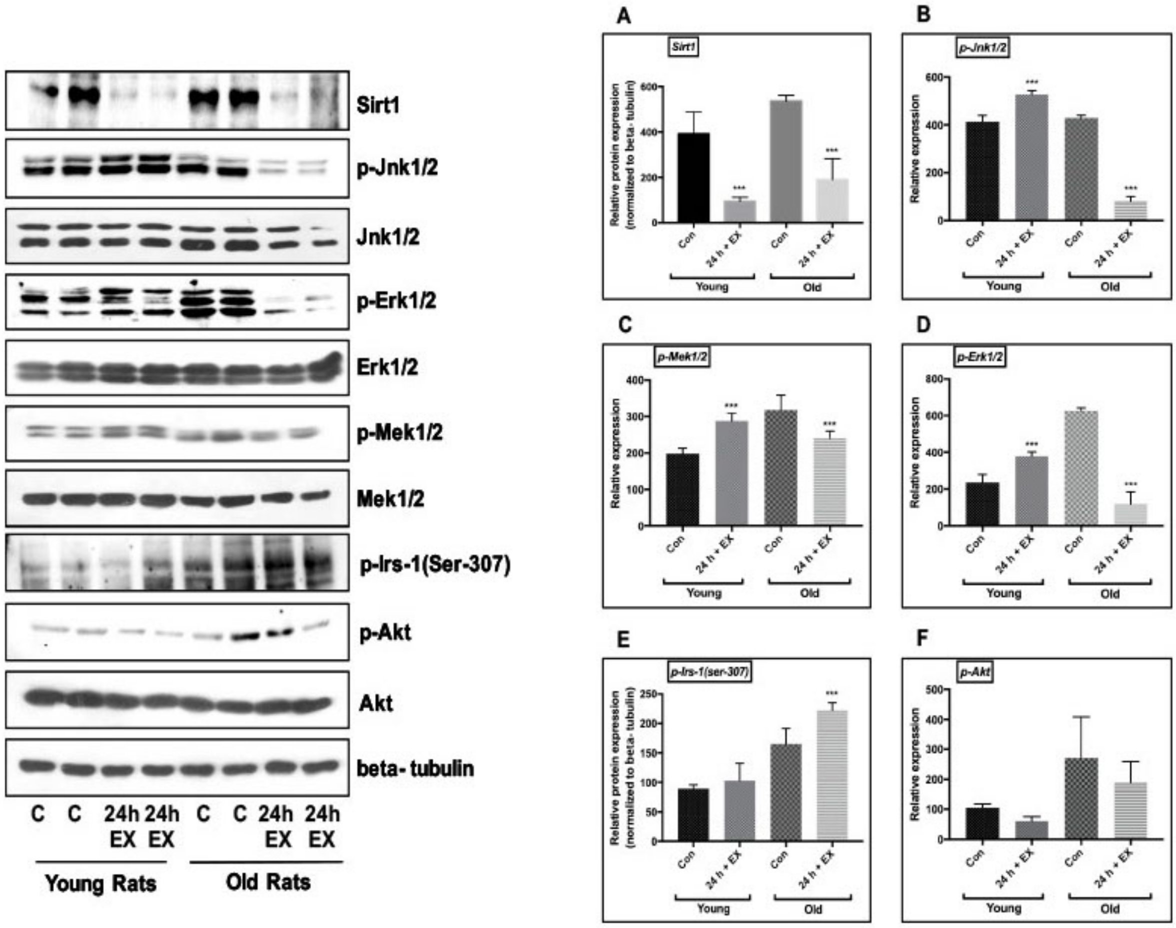


Fig. 4 Sirt1 inhibition with EX-527 treatment inhibited MAPK/Akt signaling cascade in aged experimental rats after I/R. Control and experimental brains were collected at 24 h after EX-527 treatment. An equal amount of total protein sample (75g) was probed by western blots to analyze the activity of Sirt1 and phosphorylation status of p-Jnk1/2, p-Mek1/2, p-Erk1/2, p-Irs-1, and p-Akt. Respective total proteins and beta-tubulin were served as the loading control (p-Jnk1/2, p-Mek1/2, p-Erk1/2, and p-Akt), while Sirt1 and p-Irs-1 were normalized to beta-tubulin. (**A-F**) Graphs represent the changes in expression of Sirt1/MAPK/Akt pathway after EX-527 treatment. The densitometry values are represented as mean ± SD. $*p<0.05$ versus control brain. ***Con*** - control; ***24 h EX*** – 24 h MCAO + EX-527; ***I/R*** – Ischemia-reperfusion.

Figure. .5:

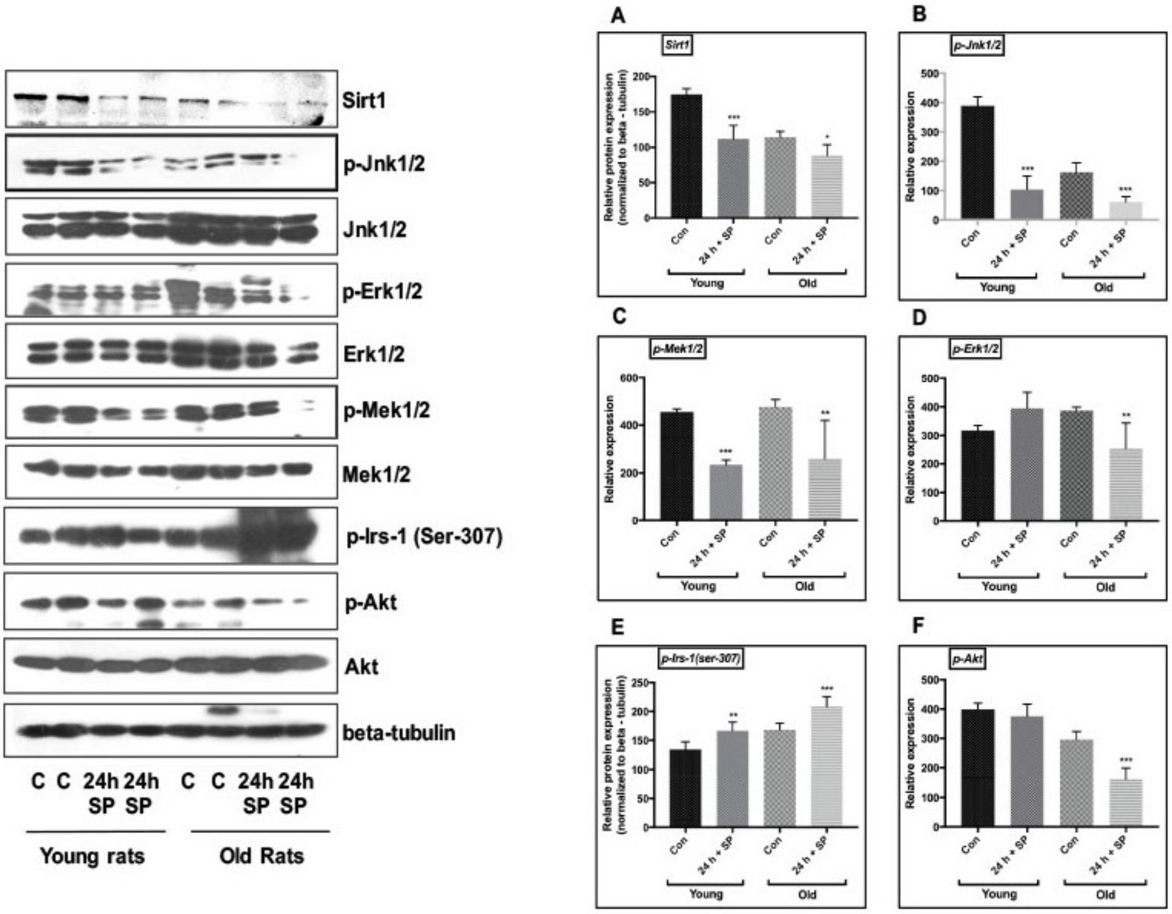


Fig. 5 Jnk inhibition with SP600125 treatment inhibited MAP Kinase signaling cascade in aged experimental rats after I/R. Control and experimental brains were collected at 24 h after SP600125 treatment. An equal amount of total protein sample (75g) was probed by western blots to analyze the activity of Sirt1 and phosphorylation status of p-Jnk1/2, p-Mek1/2, p-Erk1/2, p-Irs-1, and p-Akt. Respective total proteins and beta-tubulin were served as the loading control (p-Jnk1/2, p-Mek1/2, p-Erk1/2, and p-Akt), while Sirt1 and p-Irs-1 were normalized to beta-tubulin. (**A-F**) Graphs represent the changes in expression of Sirt1/MAPK/Akt pathway after SP600125 treatment. The densitometry values are represented as mean ± SD. ∗p<0.05 versus control brain. ***Con*** - control; ***24 h SP*** – 24 h MCAO + SP600125; ***I/R*** – Ischemia-reperfusion.

Figure. 6:

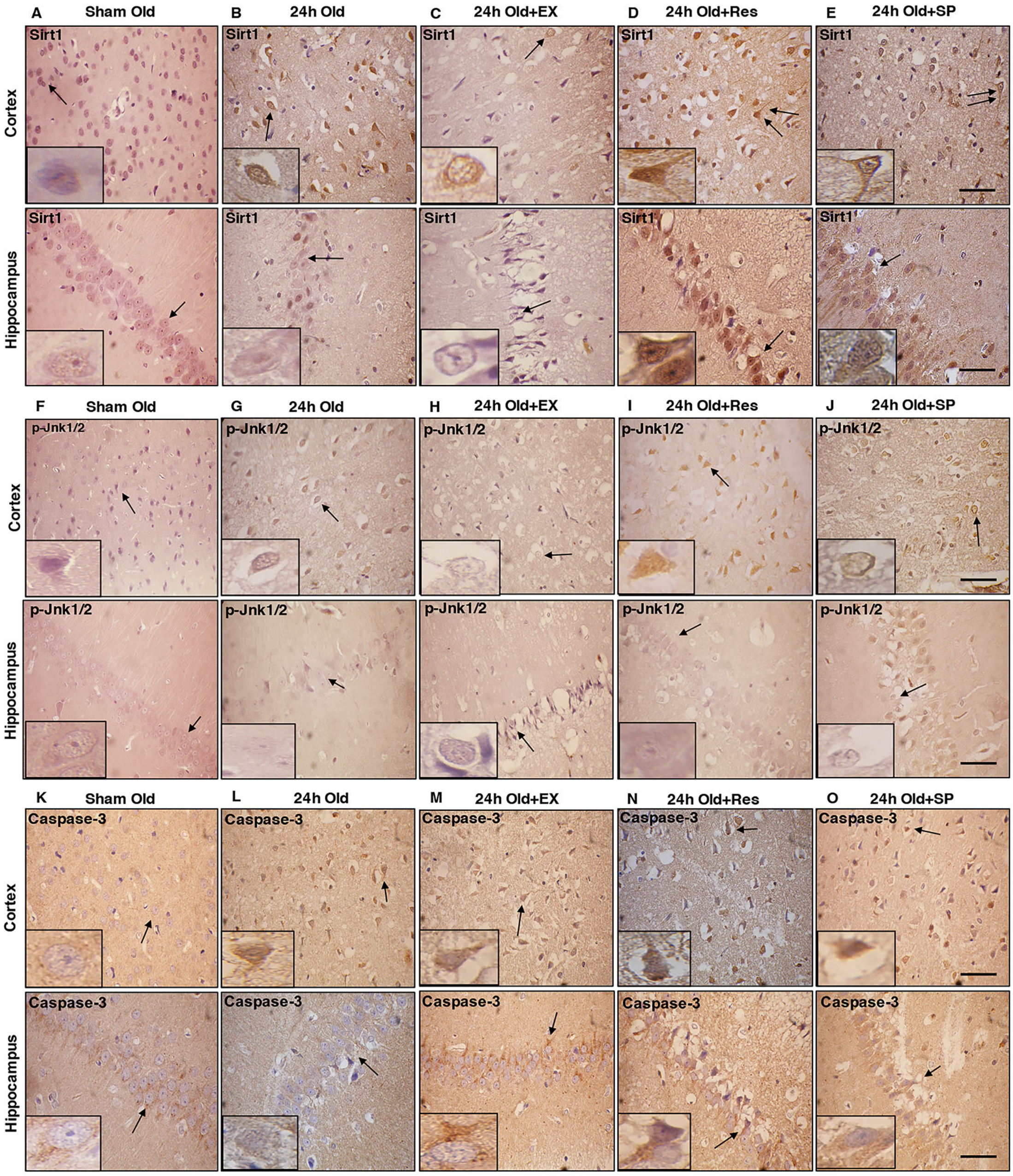


Fig. 6 Subcellular localization of Sirt1, p-Jnk1/2, & caspase-3 in aged experimental rats following I/R. Aged rat brains were paraffin embedded and sectioned into 5-10 µm thick slices. (A-E) Immunohistochemistry staining for Sirt1 in the brains of aged rat cortex and hippocampus neurons from control as well as brains from 24 h after MCAO, EX-527, resveratrol, and SP600125 treatment. Black arrow represents changes in Sirt1 immunoreactivity for the respective group. (F-J) Immunoreactivity of p-Jnk1/2 in the brains of aged rat cortex and hippocampus neurons from control as well as brains from 24 h after MCAO, EX-527, resveratrol, and SP600125 treatment. Black arrow represents changes in p-Jnk1/2 immunoreactivity for the respective group. (K-O) Immunoreactivity of total/cleaved caspase-3 in the brains of aged rat cortex and hippocampus neurons from control as well as brains from 24 h after MCAO, EX-527, resveratrol, and SP600125 treatment. Black arrow represents changes in total/cleaved caspase-3 immunoreactivity for the respective group. (magnification 400X; Scale Bar - 50µm). The neuronal nucleus is visualized with Hematoxylin counterstain. ***Sham*** – Control; ***24 h old*** – 24 h MCAO; ***24 h Old + EX*** – 24 h MCAO + EX-527; ***24 h Old + Res*** – 24 h MCAO + Resveratrol; ***24 h Old + SP*** – 24 h MCAO + SP600125; ***I/R*** – ischemia – reperfusion.

Figure. 7:

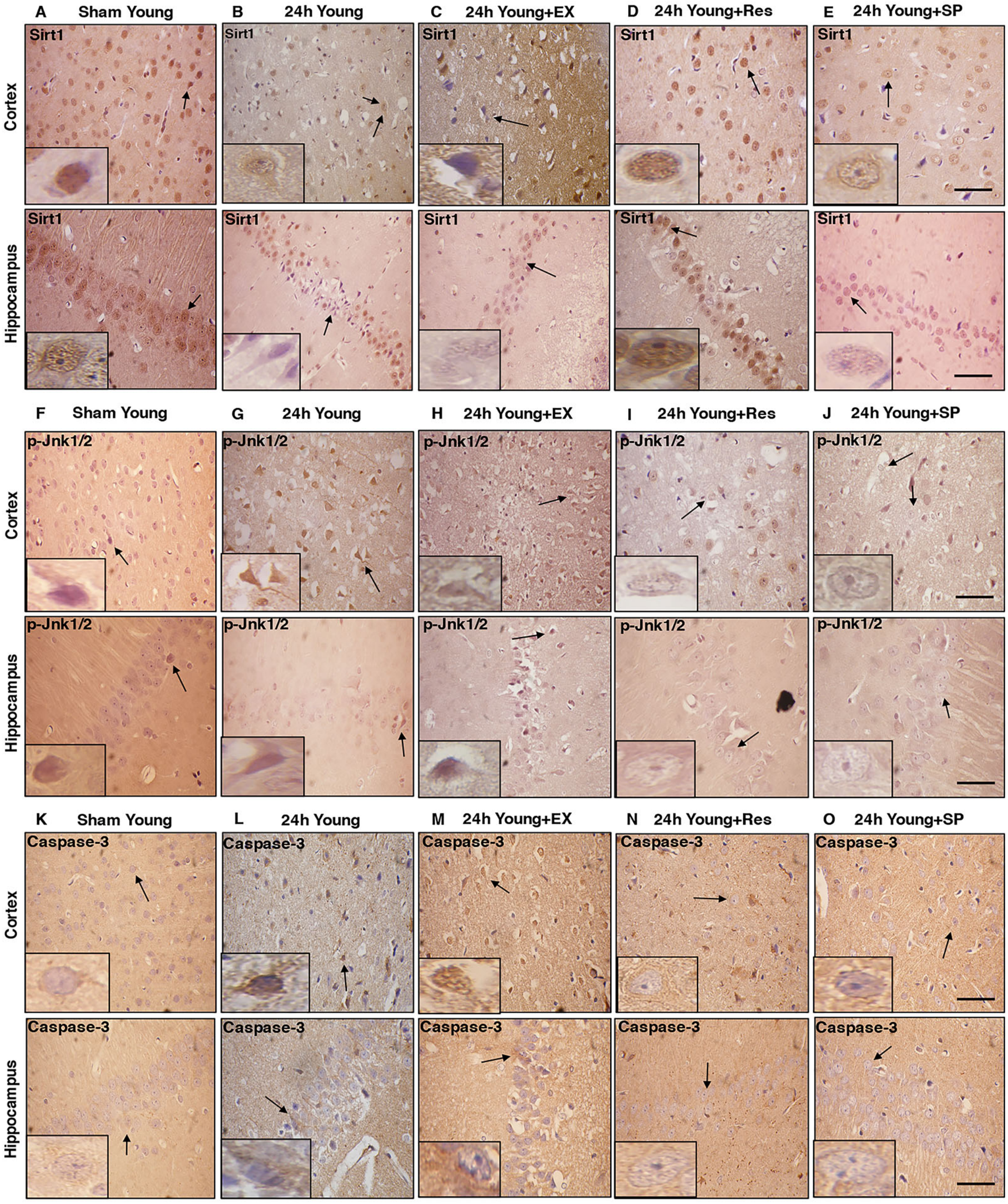


Fig. 7 Subcellular localization of Sirt1, p-Jnk1/2, & caspase-3 in the brains of young experimental rat following I/R. young rat brains were paraffin embedded and sectioned into 5-10 µm thick slices. (A-E) Immunohistochemistry staining for Sirt1 in the brains of young rat cortex and hippocampus neurons from control as well as brains from 24 h after MCAO, EX-527, resveratrol, and SP600125 treatment. Black arrow represents changes in Sirt1 immunoreactivity for the respective group. (F-J) Immunoreactivity of p-Jnk1/2 in the brains of young rat cortex and hippocampus neurons from control as well as brains from 24 h after MCAO, EX-527, resveratrol, and SP600125 treatment. Black arrow represents changes in p-Jnk1/2 immunoreactivity for the respective group. (K-O) Immunoreactivity of total/cleaved caspase-3 in the brains of young rat cortex and hippocampus neurons from control as well as brains from 24 h after MCAO, EX-527, resveratrol, and SP600125 treatment. Black arrow represents changes in total/cleaved caspase-3 immunoreactivity for the respective group. (magnification 400X; Scale Bar - 50µm). The neuronal nucleus is visualized with Hematoxylin counterstain. ***Sham Young*** – Control***; 24 h young*** – 24 h MCAO; ***24 h Young + EX*** – 24 h MCAO + EX-527; ***24 h Young + Res*** – 24 h MCAO + Resveratrol; ***24 h Young + SP*** – 24 h MCAO + SP600125; ***I/R*** – ischemia-reperfusion.

Figure. 8:

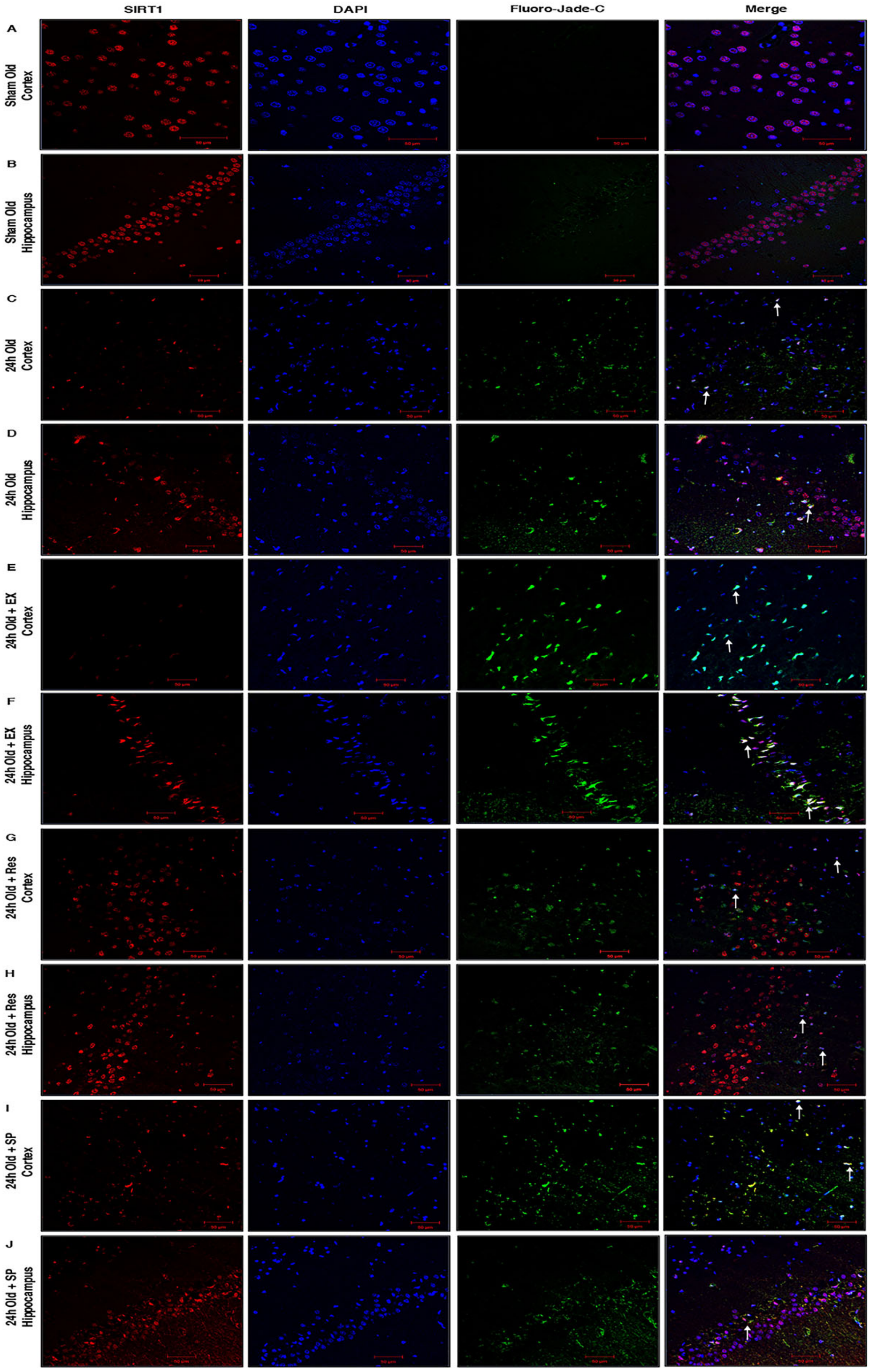


Fig. 8 Immunofluorescence labeling of Sirt1 in brains of the aged experimental rat after I/R. Triple immunofluorescence staining is used to probe for Sirt1 (Red), DAPI (Blue), and Fluoro Jade-C (Green) to assess neurodegeneration in cortex and hippocampus neurons for control as well as brains from 24 h after MCAO, EX-527, resveratrol, and SP600125 treatment. White arrows indicate co-localization of FJ-C with Sirt1 and DAPI (Scale Bar - 50µm). ***Sham old*** – Control; ***24 h old*** – 24 h MCAO; ***24 h Old + EX*** – 24 h MCAO + EX-527; ***24 h Old + Res*** – 24 h MCAO + Resveratrol; ***24 h Old + SP*** – 24 h MCAO + SP600125; ***I/R*** – ischemia reperfusion.

Figure. .9:

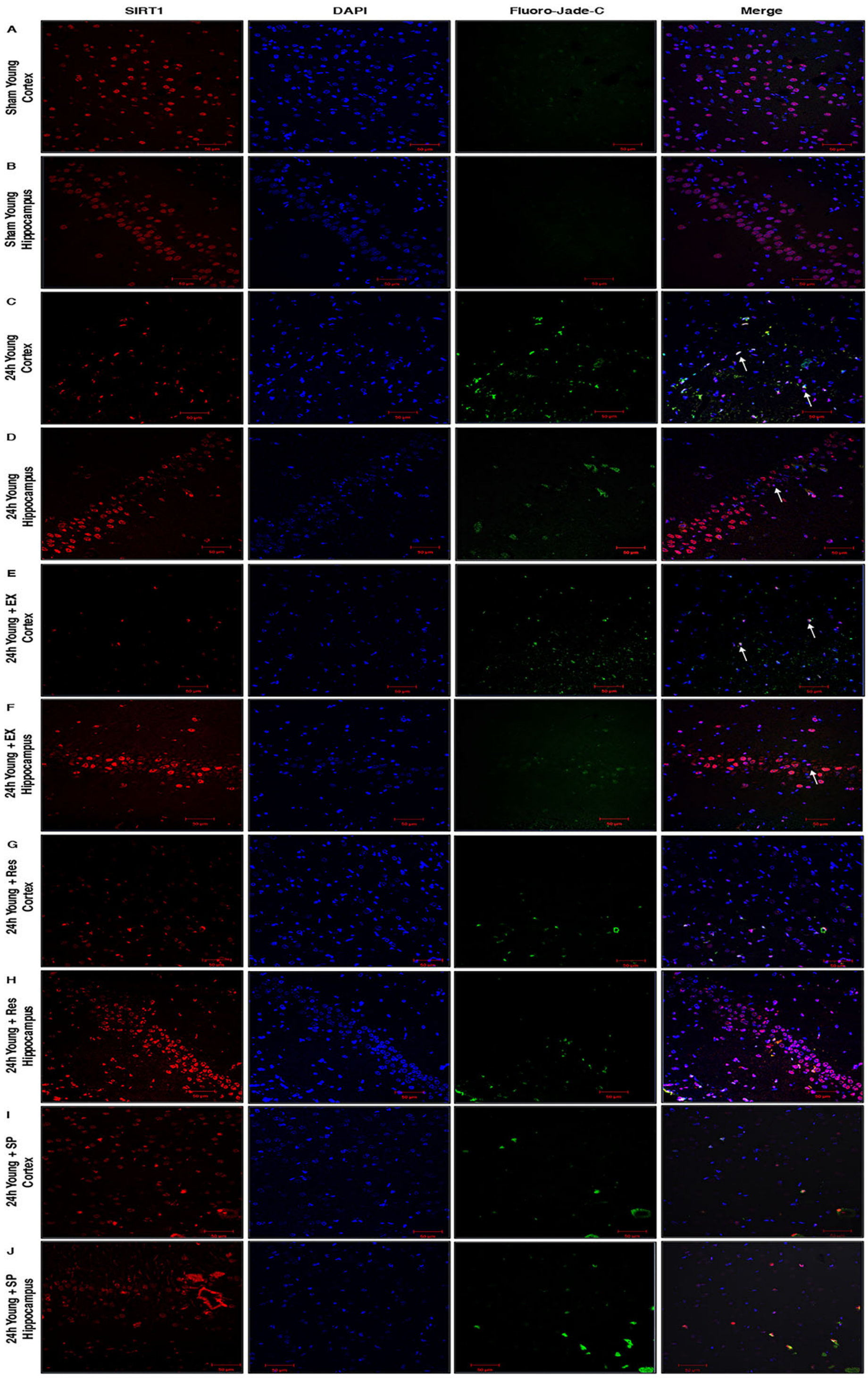


Fig. 9 Immunofluorescence labeling of Sirt1 in brains of the young experimental rat after I/R. Triple immunofluorescence staining is used to probe for Sirt1 (Red), DAPI (Blue), and Fluoro Jade-C (Green) to assess neurodegeneration in cortex and hippocampus neurons for control as well as brains from 24 h after MCAO, EX-527, resveratrol, and SP600125 treatment. White arrows indicate co-localization of FJ-C with Sirt1 and DAPI (Scale Bar - 50µm). ***Sham Young*** – Control; ***24 h young*** – 24 h MCAO; ***24 h Young + EX*** – 24 h MCAO + EX-527; ***24 h Young + Res*** – 24 h MCAO + Resveratrol; ***24 h Young + SP*** – 24 h MCAO + SP600125.

Figure. 10:

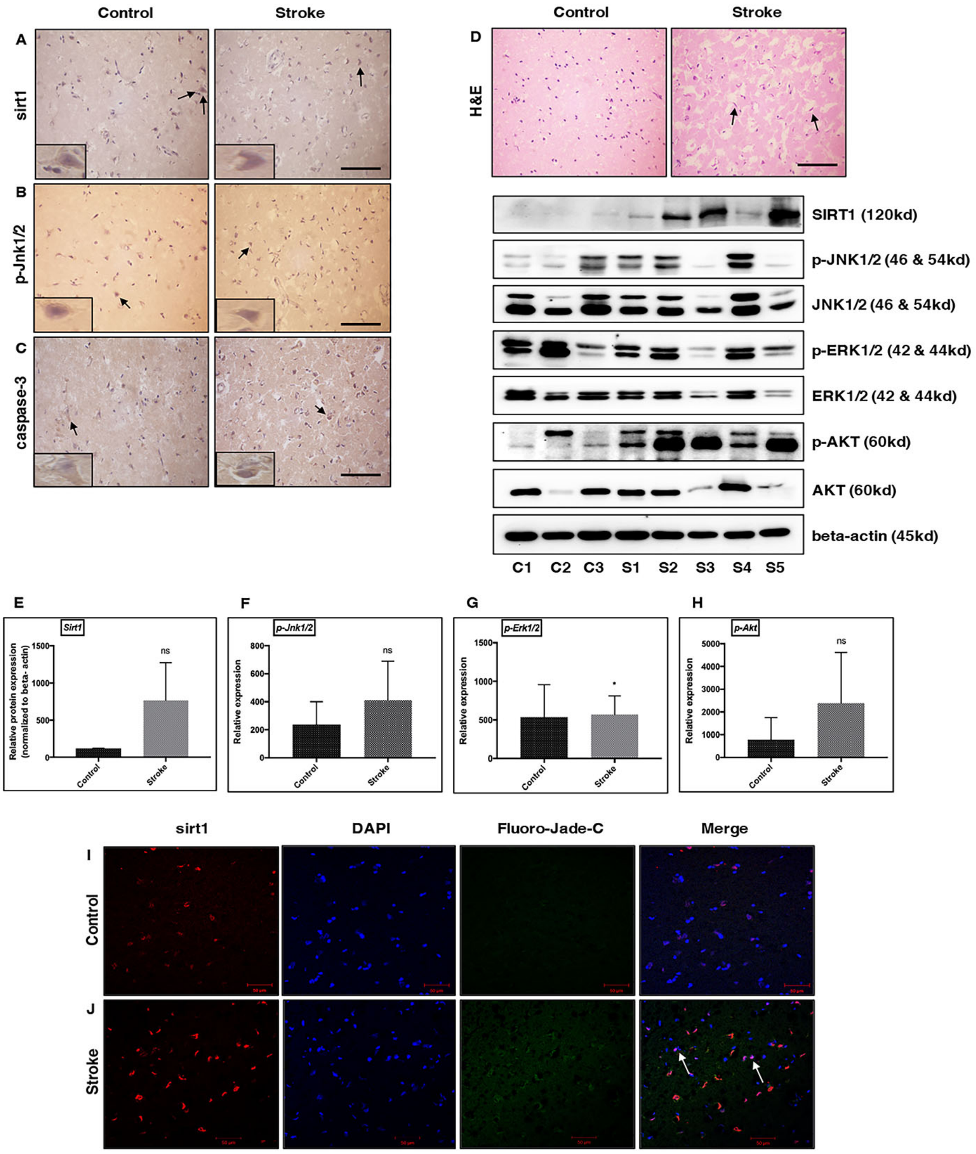


Fig. 10 Effect of ischemia on the expression of Sirt1/MAPK/Akt signaling pathway in Post-mortem human brain tissue. Human control, as well as stroke brains, were paraffin embedded and sectioned into 5-10µm thick slices. (A, B, & C) immunoreactivity of Sirt1, p-Jnk1/2, and total/cleaved caspase-3 in control and stroke human brain. Black arrow represents changes in immunoreactivity from respective group. Hematoxylin counterstain used for visualization of neuron nucleus (magnification 400X; Scale Bar - 50µm). (D) Histopathological changes were observed using H&E staining (magnification 400X; Scale Bar - 50µm). An equal amount of total protein sample (50g) from Control (n=3) and Stroke brain (n=5) were probed by western blots to analyze the expression of Sirt11 and phosphorylation status of p-Jnk1/2, p-Erk1/2, and p-Akt. Respective total proteins and beta-actin were served as the loading control (p-Jnk1/2, p-Erk1/2, and p-Akt), while Sirt1 was normalized to beta-actin. (E-H) represents an increase in Sirt11, p-Jnk1/2, p-Erk1/2, and p-Akt protein expression in human stroke brain compared to respective healthy control Data represented as mean ± SD. $*p<0.05$ versus control brain (p-Erk1/2). (I & J) Triple immunofluorescence labeling is used to probe for Sirt1 (Red), DAPI (Blue), and Fluoro Jade-C (Green) in control and stroke human brains. The representative image has shown the number of Fluoro Jade-C positive cells that co-localized with in stroke brain compared to control (White arrows), (Scale Bar - 50µm). ***C1 - C3*** – Human Control; ***S1 – S5*** – Human Stroke brain.

Figure. 11:

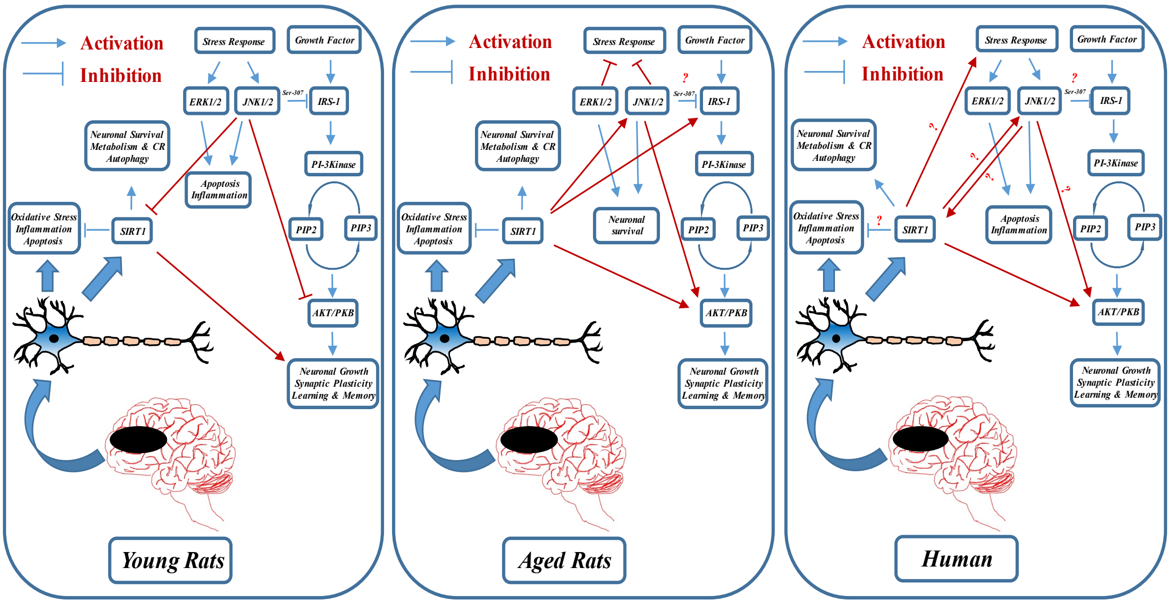


Fig. 11 Role of Sirt1/MAPK/Akt signaling pathway in rodent & human. Schematic representation of the changes in expression of Sirt1/MAPK/Akt signaling pathway in rodent and human brain following ischemia.

Table. 1: Summary of the patient's information.

Case	Age/Sex	PMI	Anatomical Area
1	42/M	2 h 30 min	Orbito frontal cortex (Normal)
2	44/M	19 h 15 min	Insular (Normal)
3	35/M	12 h	Left orbito frontal (Normal)
4	35/M	12 h	Right orbito frontal infarct
5	43/M	4 h	Right MCA territory infarct
6	65/F	12 h 30 min	Necrotic tissue right infarct
7	23/F	16 h 40 min	Hemorrhagic infarct
8	35/F	24 h	Right parieto-occipital infarct
9	19/F	NA	Left frontal infarct
10	25/F	5 h	Left temporal infarct
11	25/F	NA	Right temporal infarct
12	38/F	15 h 15 min	Right temporal infarct
13	22/F	NA	Right fronto parietal infarct

MCA–Middle Cerebral Artery; M–Male; F–Female; PMI–Post-mortem interval.

4. Discussion:

This study, for the first time demonstrated brain expression and activity of Sirt1/Jnk1/2/Erk1/2/MAPK/Akt pathway and its involvement in the regulation of stress-mediated apoptotic brain cell death in the brains of rat and human after I/R. The present study showed that, in aged rats, Sirt1 mediated neuroprotection is dependent on MAP Kinase pathway, and Sirt1 modulated the Irs-1/Akt pro-survival pathway in aged rats following I/R. In contrast, we observed an increase in Sirt1 and MAPK activity in human stroke brains compared to healthy controls, which may involve in the activation of caspase-3 mediated apoptotic cell death. Our results in young experimental rats were in line with previous reports.

Sirt1 is profoundly expressed in the brain and is involved in neuronal development, learning, and memory (Ramadori et al. 2008a), (Li et al. 2013). Sirt1 is a mediator of caloric restriction-induced longevity and known for protection against aging (Cohen et al. 2004). One of the neuroprotective mechanism thought to be controlled by Sirt1 during stroke in young experimental model is inhibition of caspase-3 dependent apoptosis (Yan et al. 2013a). Our study has shown decreased Sirt1 activity in brains of experimental rat following ischemia. Recent findings in humans reported that serum levels of Sirt1 are significantly higher in acute ischemic stroke patients compared to healthy control (Liang et al. 2019). Our results have shown that in humans, expression of Sirt1 was upregulated in stroke brains compared to the healthy controls.

Sirt1 exhibits both neuroprotective and neurodegenerative functions (Chong et al. 2005), (Gan and Mucke 2008). Various factors may involve in the regulation of Sirt1 functional outcome, including, the intensity of the stress stimuli, the availability of NAD pool (Donmez 2012a), and its subcellular localization. Cytosol-localized Sirt1 functions as pro-apoptotic factor (Jin et al. 2007), while nuclear-localized Sirt1 functions as an anti-apoptotic factor (Hisahara et al. 2008a), (Tong et al. 2013). Sirt1, being a histonal deacetylase, found to be localized in the nucleus (Michishita et al. 2005). Recent findings have shown that Sirt1 is mainly confined to the cytosol (Li et al. 2008). Our results show that Sirt1 in the infarcted brain is characterized by weak nuclear and strong cytoplasmic immunoreactivity, while in control brains Sirt1 immunoreactivity was more in nucleus. We observed more Sirt1 immunoreactive cytoplasm and cell processes of neurons in brains of aged experimental rat compared with brains of young experimental rat after I/R. The reason mainly due to age-induced increase in apoptosis (He et al. 2006), which may trigger increased cytoplasmic translocation of Sirt1 from the nucleus of neurons after I/R. In humans, Sirt1 immunoreactivity is detected in the nucleus. The nuclear immunoreactivity of Sirt1 after stroke in humans may suggest that Sirt1 activation is required to prevent neuronal cells surrendering to ischemic death.

MAP Kinase family proteins trigger various intracellular pathways involved in neuroprotection and neuronal cell death. It has been evident that MAP Kinase superfamily, including Erk1/2/MAPK, and Jnk1/2/MAPK is highly expressed in rat brain during ischemia (D. C. Wu et al. 2000). Pharmacological inhibition of Jnk1/2MAPK activity has been involved in postischemic neuroprotection in young experimental stroke animal models (Borsello et al. 2003), (Guan et al. 2006). Jnk1/2 is thought to engage in ischemic apoptosis through regulation of mitochondrial apoptotic signaling pathway and caspase-3 activation (Gao et al. 2005b), but Jnk phosphorylation during I/R injury does not always lead to apoptotic neuronal death (Herdegen et al. 1998), and Jnk/MAPK pathway offers neuroprotection during ischemic preconditioning (Pang et al. 2015). More importantly, Jnk is required for maintaining the cytoskeletal integrity of neuronal cells during maturation and aging of the CNS (Chang et al. 2003). Further, protective nature of Jnk against cardiac ischemia is well documented where Jnk confers protection against acute ischemia via Sirt1 dependent manner (Vinciguerra et al. 2012). Even though Erk is an essential component in learning and memory formation, inhibition of Mek, the upstream activator of Erk, reduce neuronal loss in models of ischemia (Sweatt 2004), (Namura et al. 2001). Our study has shown that the phosphorylation status of Jnk1/2/Erk1/2/MAP Kinases was decreased in aged rats following a stroke, while, in humans, Jnk1/2/Erk1/2/MAP Kinase phosphorylation is upregulated in stroke brains compared to the controls.

Caspase-3 is believed to be one of the vital effector caspases involved in the apoptotic cell death. A substantial body of evidence has shown activation of caspase-3 mediated apoptosis found to be involved in the death of neurons during ischemia in experimental models and human brain (Sairanen et al. 2009). Our results were by the existing evidence. We found increased caspase-3 immunoreactivity in experimental rat and human stroke brains. The inconsistency in Sirt1/MAPK pathway between aged and young experimental rat models and human maybe due to various pathways and mechanisms that are mediated by Sirt1 and MAP Kinases and the context-dependent changes in these pathways.

Resveratrol is proven to be neuroprotective during ischemia (Sinha, Chaudhary, and Gupta 2002a). Resveratrol produces a variety of health benefits in Sirt1 dependent manner (Raval, Dave, and Perez-Pinzon 2006). Resveratrol promotes adult neurogenesis in sub ventricular zone and hippocampus via upregulation of Sirt1 expression (Saharan, Jhaveri, and Bartlett 2013).

Resveratrol pre-treatment protects CA1 hippocampal neurons in Sirt1 dependent manner (Della-Morte et al. 2009). Recent findings demonstrated that resveratrol induced protective effects are also mediated by MAP Kinases in Sirt1 dependent manner during various CNS disorders including stroke. Acute resveratrol treatment modulates MAPK pathway in ischemia and alcohol-induced neurodegeneration via Sirt1 dependent manner (Shin et al. 2012), (Gu et al. 2018). Moreover, resveratrol mediated functional protection against nigrostriatal pathway injury-induced apoptosis is by modulation of c-JNK signaling (D. Li et al. 2017). We observed a substantial increase in the phosphorylation Jnk1/2/Erk1/2/MAP Kinase on Sirt1 activation. We did not find any decrease in caspase-3 immunoreactivity, where resveratrol failed to show a striking enhancement in protecting the aged brain from caspase-3 mediated neuronal apoptosis. The blunt in response to acute resveratrol treatment in aged rats could be due to various neurochemical changes in the aged brain, including, delayed response to recovery, increased cytogenesis and apoptosis (Popa-Wagner et al. 2007). Further, we observed a significant decrease in MAP Kinase phosphorylation and an increase in neuronal death on Sirt1 inhibition with EX-527 in aged rats. Resveratrol treatment abolished the effects of EX-527. Interestingly, Jnk1/2/MAPK inhibition with SP600125 decreased the phosphorylation of Mek1/2/MAPK & Erk1/2/MAPK but failed to alter Sirt1 expression. Our results were disagreeing with previous findings that Jnk/MAPK modifies the expression of Sirt1 (Gao et al. 2011). Moreover, we found increase in caspase-3 mediated apoptotic cell death in aged rats in the presence of Jnk1/2 inhibitor.

Irs-1/Akt and Sirt1/Jnk1/2 represent two parallel pathways. The interaction between these two pathways could be positive or negative. Sirt1 and Jnk1/2 share various molecular downstream targets of Irs-1/PI3/Akt pathway. Jnk1/2 phosphorylates Irs-1 at ser-307, thereby negatively regulate Akt (Aguirre et al. 2000). Akt activation by Sirt1 is necessary for its phosphorylation (Li et al. 2013), (Sundaresan et al. 2011) and Akt affects cell survival and metabolism through the Irs-1/PI3K pathway. Activation of Akt promotes neuronal survival during ischemia (Ohba et al. 2004). Resveratrol protected hippocampal neuronal cells from oxidative stress via Akt dependent manner (Zamin et al. 2006), (Fukui, Choi, and Zhu 2010). We observed the increased activity of Akt and decrease in Irs-1 phosphorylation at ser-307 in aged rats on resveratrol treatment and abolished the effects of EX-527 and SP600125. In human stroke brains phosphorylation of Akt was increased compared to control. This study concludes that phosphorylation status of Jnk1/2/Erk1/2/MAP Kinase and Akt pathway are regulated by Sirt1 in aged experimental rat and human brains after HI. Sirt1/ Jnk1/2/Erk1/2MAPK/Akt signaling cascade may represent a potential target for therapeutic interventions after HI. Further experimental studies will be needed to get a better understanding of Sirt1/ Jnk1/2/Erk1/2/MAPK/Akt pathway and its modulation on stroke outcome in humans.

Chapter-2

Delayed activation of Sirt1 induced autophagy promotes astrogliosis, infarct in rat and up-regulation of Sirt1/autophagy network in human.

1 Introduction:

Sirt1 is a class-III histonal, and non-histonal deacetylase depends on NAD for its enzymatic activity. Sirt1 is proven to target a wide range of nonhistone proteins, which mediate central aspects of cellular stress and survival (S Imai et al. 2000), (Yeung et al. 2004a), (Vaziri et al. 2001a). A substantial amount of evidence supports that activation of Sirt1 promotes neuronal survival against a variety of neurological disorders, including cerebral ischemia (Donmez 2012a). Sirt1 can influence autophagy induction directly or indirectly under the conditions of nutrient starvation/cellular stress, and Sirt1 induced autophagy plays an essential role in metabolism and resistance to cellular stress (In et al. 2008).

Autophagy is a conserved catabolic process that degrades damaged cytosolic organelles and protein aggregates for the regulation of neuronal proteostasis (Cai et al. 2019). Autophagy helps in support neuronal plasticity and blood-brain barrier integrity (Yan et al. n.d.), (Fang et al. 2015). Failure in basal autophagy has been implicated in various human neurodegenerative disorders (Menzies et al. 2017). The role of autophagy after ischemic stroke and its contribution to neuronal fate remains elusive. Recent findings have reported dramatic activation of autophagy mechanism following ischemia (Chung, Choi, and Park 2018), (Jiang et al. 2017). However, autophagy can function both pro-survival as well as pro-death mechanisms after stroke (Liu et al. 2018), (Zhou et al. 2017).

The polyphenol curcumin is an active constituent derived from curcumin longa. The protective nature of curcumin is well documented against various neurodegenerative disorders (Monroy, Lithgow, and Alavez 2013), and the beneficial effects of curcumin are mainly derived from its anti-inflammatory and anti-apoptotic properties against cerebral ischemia (W. Li, Suwanwela, and Patumraj 2017). Recent findings suggest that curcumin can promote Sirt1 expression and also modulates autophagy mechanism following ischemia (Miao et al. 2016), (Huang et al. 2018). Astrocytes are the vital constituents of the neurovascular unit, which involves diverse aspects of central nervous system functions. Activation of astrocytes is reported after ischemia-reperfusion, and their activation promotes glial scar formation whereby astrocytes undergo various molecular and morphological changes (Liu and Chopp 2016). Importantly, curcumin is shown to inhibit astrocyte mediated glial scar formation against nervous system injury (Yuan et al. 2017).

Emerging evidence shows that some of the mediators of acute neuronal injury have a protective role during the delayed phase of ischemia. The stress-responsive Jnk is shown to promote neurovascular remodeling and recovery during the delayed stroke (Murata et al. 2012). Activation of matrix metalloproteinases (MMP) involves the degradation of extracellular matrix during acute ischemia but inhibition of MMPs during the chronic phase is shown to prevent neurovascular remodeling and functional recovery (Zhao et al. 2006). Similarly, delayed inhibition of excitotoxic NMDA receptors prevents synaptic plasticity and stroke recovery (Besancon et al. 2008). In the present study, we investigated the role of Sirt1 induced autophagy mechanism in neuronal cell death following ischemia-reperfusion (I/R) in the brains of humans and in rats from day1 (acute phase) to day7 (chronic phase). Studying Spatio-temporal changes in autophagy network in the experimental animal model and human brains may help in a rich understanding of autophagy and its involvement in stroke pathophysiology.

2 Materials & methods:

2.1 Transient Middle Cerebral Artery Occlusion (MCAO):

Young (250-300g) male Sprague-Dawley (SD) rats were randomly split into five groups: (i) Control group, (ii) MCAO group (day1 – day7), (iii) Vehicle group (day7 + saline), (iv) MCAO + Curcumin group, (v) MCAO + Curcumin + EX-527 group. All the drug administrations were performed on day7 post-ischemia. Two different treatment protocols were followed for drug administration. Curcumin alone and Curcumin + EX-527 combination was given on day7 post-ischemia. The combination therapy (curcumin + EX-527) was preferred to minimize the animal number and their suffering. Curcumin (300 mg in 5N NaOH; pH-7.4) was administered via IP route (Thiyagarajan and Sharma 2004), and EX-527 (30 µg in 1% DMSO in PBS) was administered into lateral ventricles on day7 post-stroke (Yan et al. 2013b), and after 24 h of drug administration rats were sacrificed. Curcumin and EX-527 were purchased from Sigma (USA). Focal cerebral ischemia was induced by occluding MCA as mentioned in the chapter-1- materials & methods section.

2.2 Evaluation of Neurological deficit:

The cognitive deficit was evaluated for infarct intensity by Bederson score (Bederson et al. n.d.) as mentioned in the chapter-1- materials & methods section.

2.3 Infarct volume measurement:

Brains from control and experimental rat (day1-day7, day7 + Curcumin, and day7 + Curcumin + EX-527 treatment) after stroke were stained with 1% 2,3,5- triphenyl tetrazolium chloride (TTC). The TTC staining was performed using the method as mentioned in the chapter-1, materials & methods section.

2.4 Immunoblotting:

Rats were euthanized with the overdose of carbon dioxide (CO_2) and ipsilateral brains were excised from control, day1 to day7, curcumin treatment, and curcumin+EX-527 treatment after reperfusion. An equal amount of total protein sample (75g) was probed by western blots. Total Protein extract was resolved on 10% SDS-PAGE gels. Immunoblotting was performed against Sirt1, beta-actin, Beclin-1, Atg-3, Atg-5, Atg-7, and LC-3a/b, total & phospho mTOR using the method as mentioned in the chapter-1, materials & methods section. All the primary and secondary antibodies were from Cell Signaling Technology (CST, USA).

2.5 Immunohistochemistry:

Brains of control and experimental rat (day7, day7 + curcumin, and day7 + curcumin + Ex-527 treatment after reperfusion were fixed by trans cardiac perfusion with PBS and 4% PFA. Immunohistochemistry was performed against Sirt1, and caspase-3 (pro/cleaved) using the method as mentioned in the chapter-1, materials & methods section. Brain tissue pictures were taken under the light microscope with 400 X magnification.

2.6 Haematoxylin & Eosin (H & E) staining:

Brain sections of control and experimental rat (day7, day7 + curcumin, and day7 + curcumin + Ex-527 treatment) after stroke were dewaxed in three changes of xylene followed by hydration in a decreased gradient of alcohol. After being washed with PBS, brain sections were stained with hematoxylin, followed by eosin. Sections were dehydrated, cleared in xylene, and mounted. Images were observed under the microscope for the changes in neuronal morphological under 400 X magnification.

2.7 Immunofluorescence:

The brain Sections from the brains of control and experimental rat (day7, day7 + curcumin, and day7 + curcumin+EX-527 treatment) after stroke were used for Immunofluorescence labeling to probe for LC-3a/b and astrocyte intermediate filament protein GFAP (CST, US). Immunofluorescence labeling was performed using the methods as mentioned in the chapter-1, materials & methods section.

2.8 Post-mortem human brain tissue:

An autopsy cohort of ischemic stroke (n=4) and control (n=3) human brain tissues were obtained from "Human Brain Bank, Department of Neuropathology, National Institute of Mental Health & Neurosciences (NIMHANS); Bangalore, India." All the experimental procedures on human tissues were approved by the Institutional Ethical Committee (IEC), University of Hyderabad, India. Informed consent was obtained from the family members of the patient. Patient details, including age, sex, and region of the infarct, are given in Table-1. Brain tissues from Control and stroke were embedded in paraffin following formalin fixation. Paraffin-embedded brain sections were immunostained against Beclin-1, Atg7, Sirt1 & LC-3a/b using the method as mentioned in the immunohistochemistry section.

3 Results:

3.1 Ischemia-reperfusion induced expression of Sirt1/autophagy/mTOR pathway in a time-dependent manner:

To determine the expression of the Sirt1/autophagy/p-mTOR pathway in a time-course manner after ischemia-reperfusion, we performed immunoblotting. Our results show that on 24 h after reperfusion, the expression of Sirt1 was decreased, and most of the autophagy mediators, including Beclin-1, Atg-3, Atg-5, and Atg-7, were downregulated, except LC-3a/b, which was upregulated compared with the control (Figure. 13 A-F, Sirt1<0.02; Beclin-1<0.001; Atg-3<0.001; Atg-5<0.003; Atg-7<0.001; LC-3a/b<0.001). Further, from day2 to day6 after stroke, we observed an increase in Sirt1 expression and autophagy mediators compared to day1 after stroke. Importantly, on day7 after stroke, the expression of Sirt1, and autophagy mediators were found to be decreased compared with the control (Figure. 13 A-F, Sirt1<0.001; Beclin-1<0.001; Atg-3<0.001; Atg-5<0.001). Notably, the expression of p-mTOR is inverse correlates with the autophagy mechanism.

On day1 and day7 after stroke, the expression of p-mTOR was upregulated (Figure. 13 G, p-mTOR<0.001), and from day2 to day6 after stroke, the expression of p- mTOR was decreased compared with the brains of day1 rat after stroke (Figure. 13 G).

The immunoblotting results were consistent with Sirt1 and LC-3a/b immunostaining studies, where immunoreactivity of both Sirt1 and LC-3a/b was found to be decreased on day7 post-stroke (Figure. 15 A & 16 A). Moreover, GFAP positive immunostaining has shown reactive astrogliosis, which promotes glial scar, on day7 after stroke compared with the brains of a control rat (Figure. 16 B).

3.2 The decrease in infarct and apoptotic cell death in a time-dependent manner after stroke:

Cerebral infarction was measured by TTC staining. A significant amount of infarction was observed in ipsilateral brains after 24 h of reperfusion. The decrease in infarct was seen in a time-course manner from day1 to day7 after stroke, and a substantial reduction in infarct was observed on day7 compared with day1 after stroke (Figure. 12 B). The neuronal injury was confirmed by Hematoxylin & Eosin staining on day7 after stroke. We observed neuronal loss on day7 post-stroke compared with the control (Figure. 12 A). Further Apoptotic cell death was confirmed by caspase-3 immunoreactivity (total/cleaved caspase-3). Caspase-3 immunoreactivity in control was mainly observed in the cytoplasm of cortex and hippocampus neurons, while on day7 post-stroke immunoreactivity was found in neuron nucleus and caspase-3 immunoreactivity was increased on day7 post-stroke compared with the control (Figure. 15 B).

3.3 Delayed curcumin treatment upregulates Sirt1 induced autophagy network and decreases phosphorylation of mTOR after stroke:

To study the effect of curcumin on Sirt1 induced autophagy mechanism on day7 post-stroke, immunoblotting was performed to investigate the expression of Sirt1/autophagy/ mTOR pathway and found that Sirt1 induced autophagy was upregulated and phosphorylation of mTOR was decreased on day7 after curcumin treatment compared with the vehicle (day7) (Figure. 14 A-G, Sirt1<0.001; Beclin-1<0.001; Atg-3<0.03; Atg-5<0.001; Atg-7<0.001; LC-3a/b<0.001, p-mTOR<0.001). We further performed immunostaining against Sirt1 and LC-3a/b. We found the absence of Sirt1 and LC-3a/b immunoreactivity, and our immunoblotting findings were not reflected on immunohistochemistry, which might be due to the reactive astrogliosis on day7 after curcumin treatment compared with day7 after stroke (Figure. 15 A & 16 A).

3.4 Delayed curcumin treatment promotes infarct, reactive astrogliosis, and caspase-3 mediated apoptotic cell death after stroke:

Brain sections were assessed for infarct development by TTC staining. After curcumin treatment on day7 post-stroke, infarct formation was increased compared with the brains of the experimental rat on day7 after stroke (Figure. 12 B). The presence of neurodegeneration was evaluated by H&E staining and found that curcumin treatment on day7 aggravated neuronal cell death compared to the brains of the experimental rat on day7 after stroke (Figure. 12 A). Further presence of apoptotic cell death was studied by caspase-3 immunoreactivity and found that curcumin treatment increased caspase-3 immunoreactivity on day7 after stroke compared with the brains of the experimental rat on day7 post-stroke (Figure. 15 B). Importantly we found an increase in GFAP positive reactive astrocytes with hypertrophied and ramified cell bodies on day7 after curcumin treatment compared with the brains of the experimental rat on day7 after stroke (Figure. 16 B). Our results show that curcumin treatment promoted scar formation and neurodegeneration on day7 after ischemia via Sirt1 mediated autophagy activation.

3.5 Delayed inhibition of Sirt1 expression down-regulates autophagy network, reduces reactive astrogliosis, infarct, and caspase-3 mediated apoptotic cell death after stroke:

To further understand the interaction of Sirt1/autophagy/mTOR pathway during the delayed phase of the infarct, rats were treated with the combination of curcumin + EX-527 on day7 after stroke and found that Sirt1 mediated autophagy network was down regulated and phosphorylation of mTOR was up-regulated compared with the curcumin-treated group on day7 after stroke (Figure. 14 A-G, Sirt1<0.001; Beclin-1<0.02; Atg-3<0.03; Atg-5<0.001; Atg-7<0.001; LC-3a/b<0.001; p-mTOR<0.001). The characteristic TTC staining shows that curcumin + EX-527 treatment on day7 post-stroke substantially reduced infarct volume compared with the curcumin group on day7 post-stroke (Figure. 12 B). Neurodegeneration evaluated with H&E show that curcumin + EX-527 treatment reduced neuronal loss on day7 post-stroke compared with the curcumin-treated group on day7 (Figure. 12 A). Apoptotic cell death was studied by caspase-3 immunoreactivity and found that curcumin + EX-527 treatment on day7 post-stroke reduced immunoreactivity of caspase-3 compared with the curcumin-treated group on day7 post-stroke (Figure. 15 B). Further GFAP immunostaining show reduced astrocyte ramifications, decreased reactive astrogliosis on curcumin + EX-527 treatment compared with the curcumin-treated group on day7 after stroke (Figure. 16 B). The immunostaining of Sirt1 and Lc3 illustrated that few immunoreactive cells were observed due to decreased astrogliosis in the curcumin + EX-527 group compared with the curcumin-treated group on day7 after stroke (Figure. 15 A & 16 A). Our results show that partial inhibition of Sirt1 induced autophagy network decreased neurodegeneration and reduced reactive astrogliosis during the chronic phase of the stroke.

3.6 Increase in Sirt1, Beclin-1, Atg-7, and LC3 immunostaining in post-mortem human stroke brain:

To understand the change in expression of Sirt1 and autophagy network in humans after ischemia, we immunostained brain sections against Beclin-1, Atg-7, Sirt1, and LC-3a/b. We observed an increase in Sirt1, Beclin-1, Atg-7, and LC-3a/b immunoreactivity in stroke brain when compared to healthy control (Figure. 17 A-D). Further, double immunofluorescence labeling of Sirt1 and LC3 shows that an increased number of LC3 positive cells co-localized with Sirt1 in stroke brain when compared to the healthy control (Figure. 17 E). Our results show that the Sirt1 mediated autophagy network was upregulated in the human brain after stroke.

Figures:

Figure. 12:

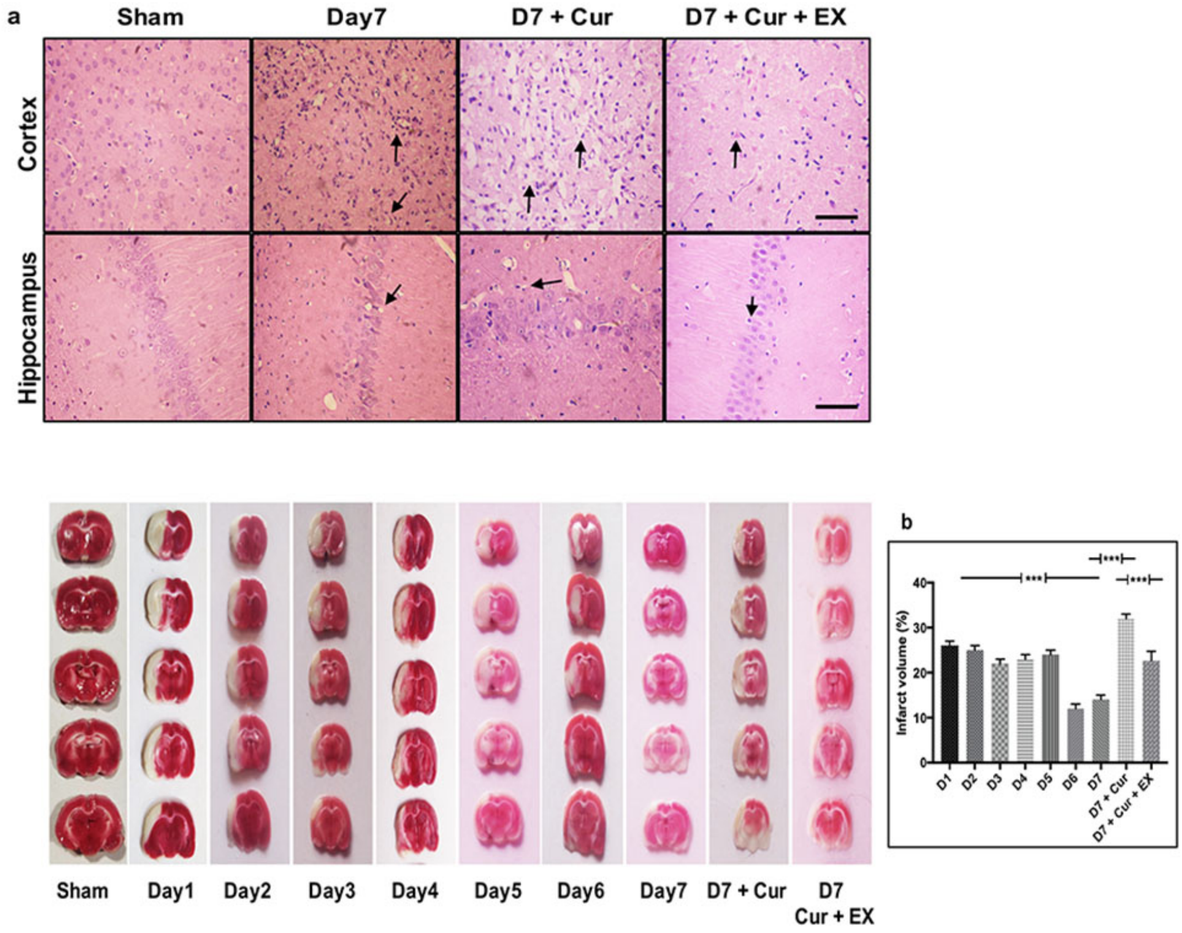


Fig. 12 Delayed curcumin treatment on day7 promoted neuronal loss after I/R. Rat brains were paraffin-embedded and sectioned into 5-10 mm thick slices (A) Representative image shows the changes in neuronal morphology of cortex and hippocampus neurons (black arrow) stained with H & E in the brains of control, day7, curcumin, and curcumin + EX-527 treatment on day7 post-stroke. (magnification 400X; Scale Bar - 50µm). (B) Representative images from the brains of control, day7, curcumin, and curcumin + EX-527 treatment on day7 post-stroke after TTC staining. The infarct volume was significantly reduced in the brains of curcumin + EX-527 treatment on day7 after stroke compared to curcumin treatment on day7 post-stroke. The data represented as a percentage of tissue loss. $*p<0.05$ versus ischemic brain on day1 after reperfusion. ***Sham*** - control; ***Day7*** – Day 7 post-stroke; ***D7 + Cur*** – Day7 post-stroke + curcumin; ***D7 + Cur + EX*** – Day7 post-stroke + curcumin + EX-527; ***I/R*** – Ischemia-reperfusion.

Figure. 13:

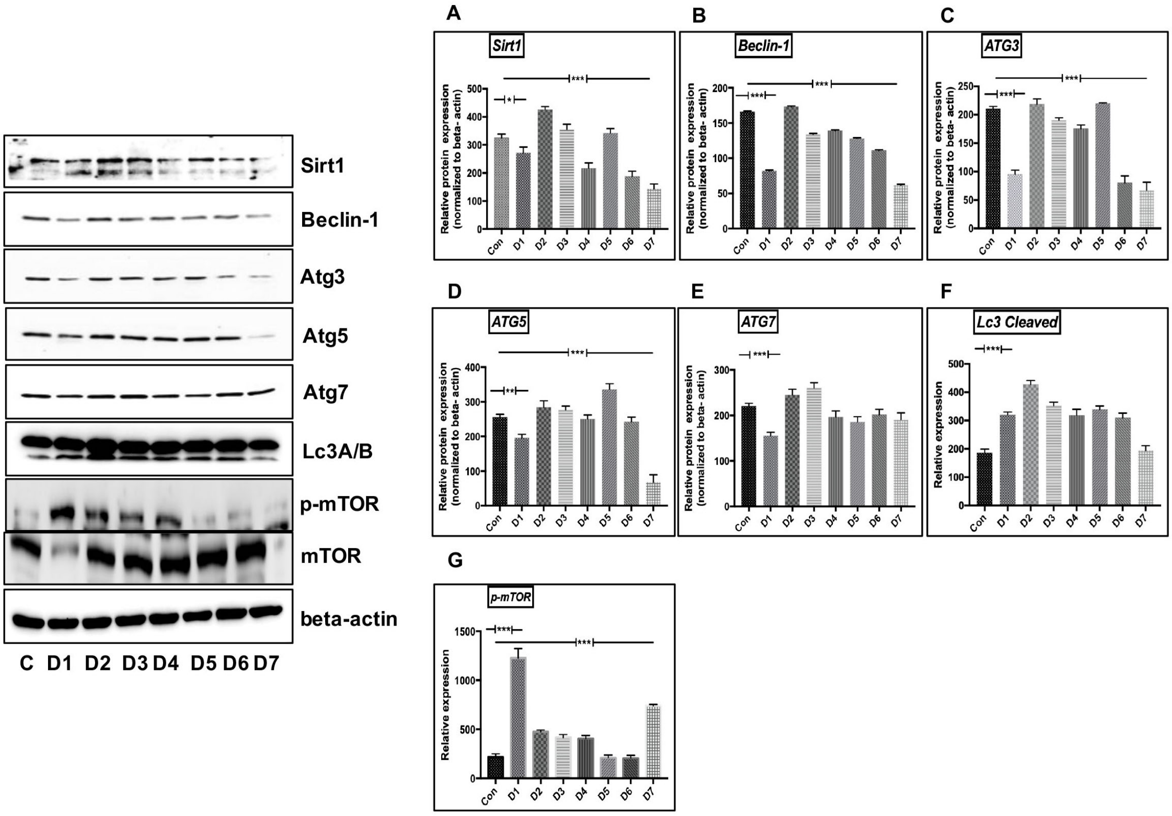


Fig. 13 Time-dependent changes in the expression of the Sirt1/autophagy/mTOR pathway after I/R. An equal amount of total protein sample (75ug) was resolved on SDS-PAGE gels. Respective total proteins and beta-actin were served as the loading control (LC-3a/b, p-mTOR), while Sirt1, Beclin-1, Atg-3, Atg-5, and Atg-7 were normalized to beta-actin. (A-G) The graphs show the time-dependent changes in the expression of the Sirt1/autophagy/mTOR signaling pathway from day1 to day7 post-stroke. The densitometry values are represented as mean ± SD. *p<0.05 versus control brain. ***C*** - control; ***D1-D7*** – Day1 to Day7 after stroke.

Figure. 14:

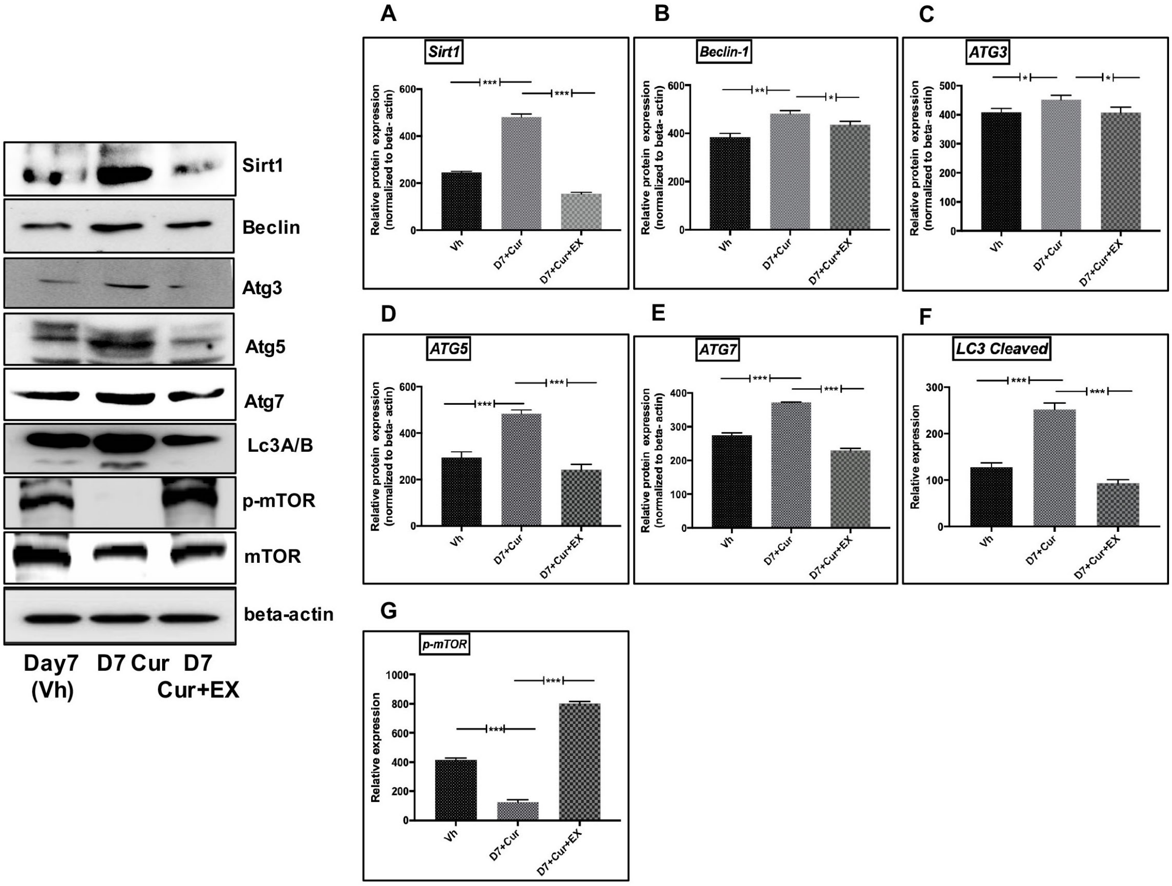


Fig. 14 Delayed curcumin treatment on day7 post-stroke up-regulates Sirt1 induced autophagy mechanism. An equal amount of total protein sample (75ug) was used for immunoblotting. Respective total proteins and beta-actin were served as the loading control (LC-3a/b, p-mTOR), while Sirt1, Beclin-1, Atg-3, Atg-5, and Atg-7 were normalized to beta-actin. (A-G) The graphs from the brains of the vehicle (day7), curcumin, and curcumin + EX-527 treatment on day7 after stroke shows that curcumin treatment promoted Sirt1 induced autophagy mechanism on day7 after stroke compared to vehicle. The brains from curcumin + EX-527 treatment show a substantial decrease in Sirt1 induced autophagy compared to curcumin treatment on day7 after stroke. The densitometry values are represented as mean ± SD. $*p<0.05$ versus control brain. ***Vh*** – Vehicle (Day7); ***D7 Cur*** – Day7 + curcumin post-stroke; ***D7 + Cur + EX*** - Day7 + curcumin + EX-527 post-stroke.

Figure. 15:

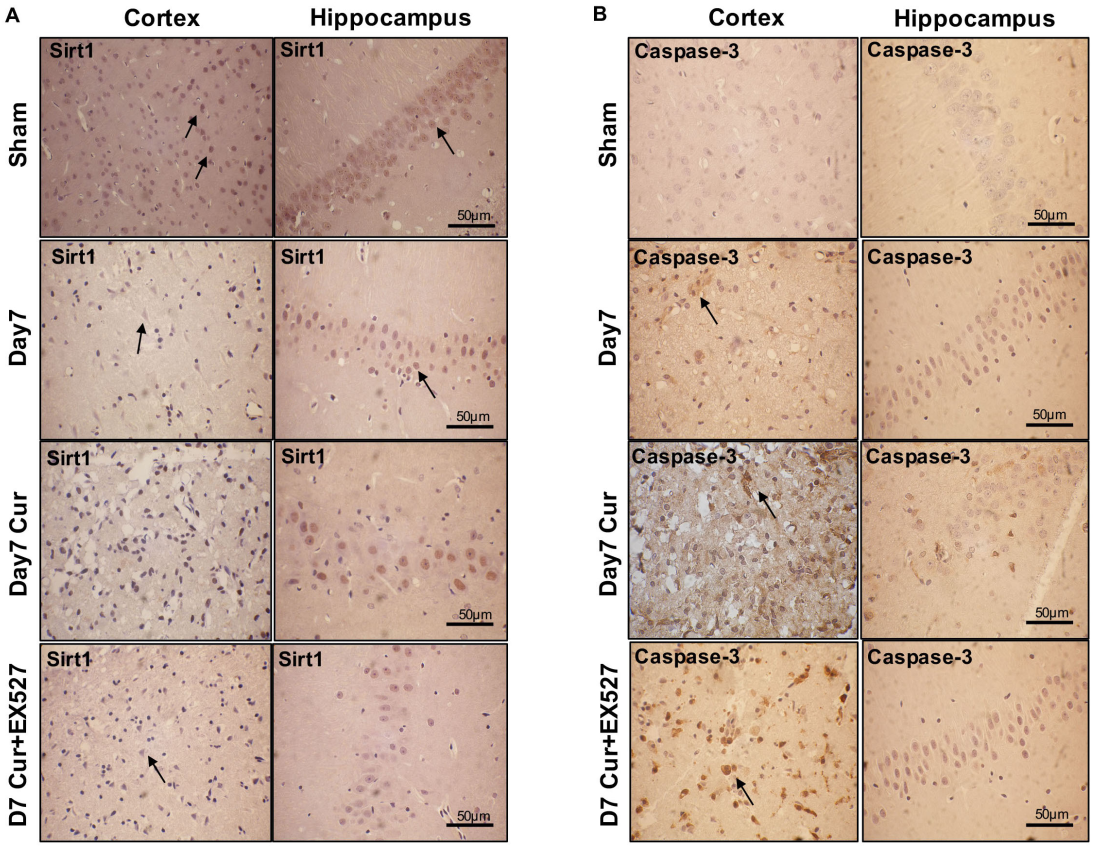


Fig. 15 Subcellular localization of Sirt1, and caspase-3 in the brains of the experimental rat after I/R. The brains from the experimental rat were paraffin-embedded and sectioned into 5-10 µm thick slices. (A) Immunohistochemistry staining for Sirt1 in the brains of rat cortex and hippocampus neurons from control as well as brains from day7, curcumin, and curcumin + EX-527 treatment on day7 after stroke. The black arrow represents changes in Sirt1 immunoreactivity for the respective group. (B) Immunohistochemistry staining for caspase-3 (pro/cleaved) in the brains of rat cortex and hippocampus neurons from control as well as brains from day7, curcumin, and curcumin + EX-527 treatment on day7 after stroke. The black arrow represents changes in caspase-3 immunoreactivity for the respective group. (magnification 400X; Scale Bar - 50µm). The neuronal nucleus is counterstained with hematoxylin. ***Sham*** - control; ***Day7*** – Day7 post-stroke; ***Day7 Cur*** – Day7 + curcumin post-stroke; ***D7 + Cur + EX527*** - Day7 + curcumin + EX-527 post-stroke.

Figure. 16:

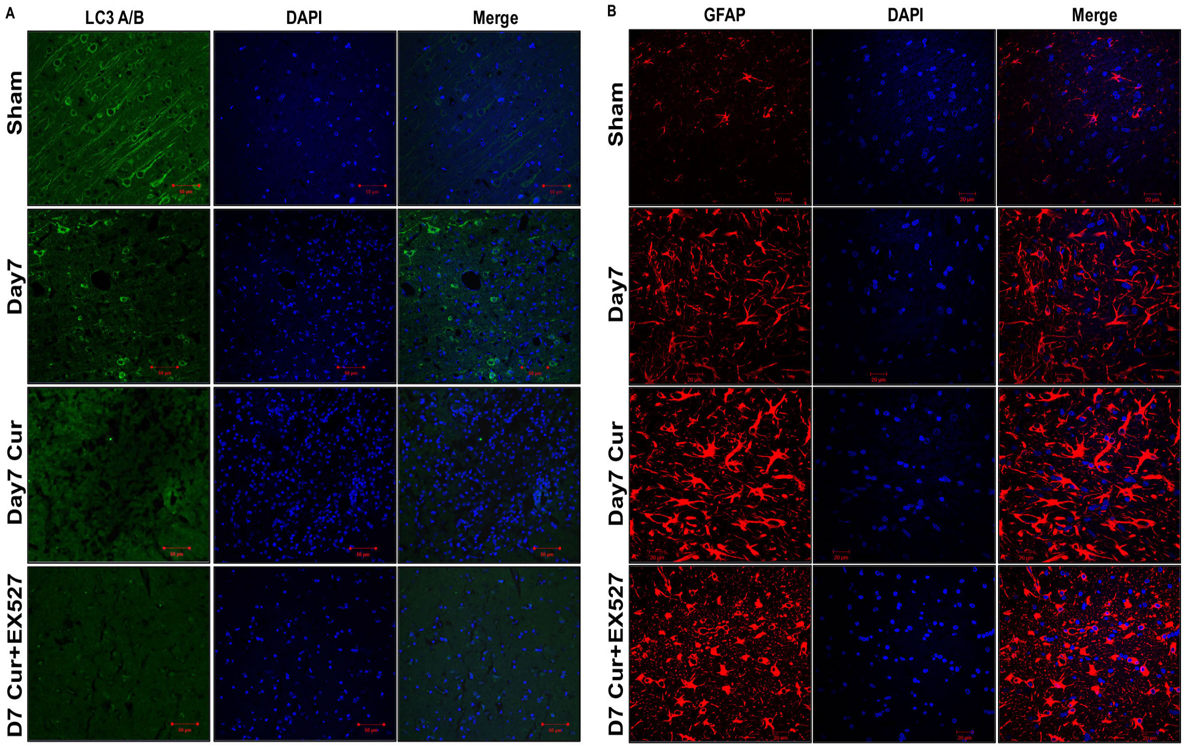


Fig. 16 Immunofluorescence labeling of LC-3a/b, and GFAP in the brains of the experimental rat after I/R. (A) Double immunofluorescence labeling for LC-3a/b (green), DAPI (blue), image shows the changes in the expression of LC-3a/b in cortex neurons from control as well as brains from day7, curcumin, and curcumin + EX-527 treatment on day7 after stroke. (B) Double immunofluorescence labeling for GFAP (red), and DAPI (blue), to assess astrogliosis in cortex neurons from control as well as brains from day7, curcumin, and curcumin + EX-527 treatment on day7 after stroke. Curcumin treatment on day7 promoted glial scar and curcumin + EX-527 treatment reduced glial scar on day7 post-stroke. (Scale Bar - 50μm).

Figure. 17:

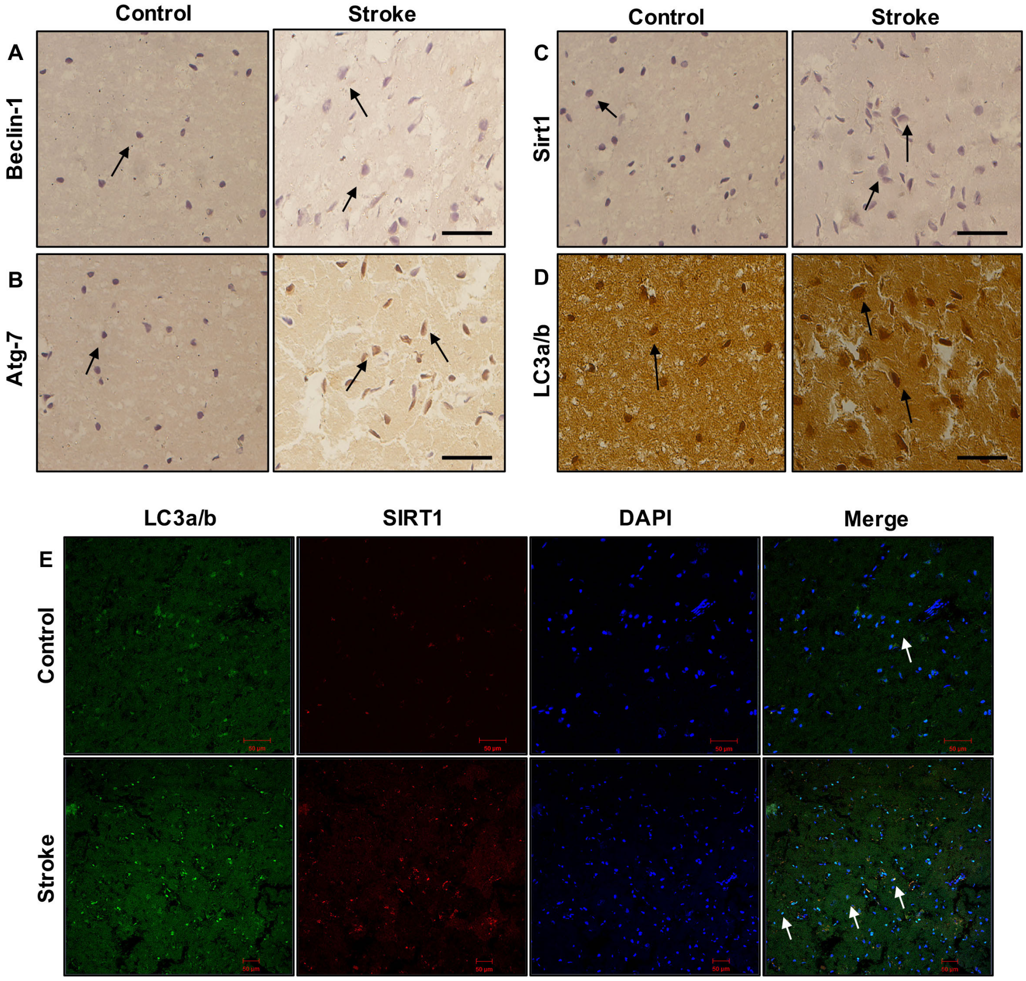


Fig. 17 The expression of Sirt1/autophagy (Beclin-1/Atg-7/LC-3a/b) signaling pathway in Post-mortem human brain tissue following ischemia. Human control, as well as stroke brains, were paraffin-embedded and sectioned into 5-10µm thick slices. (A-D) immunoreactivity of Beclin-1, Atg-7, Sirt1, and LC-3a/b in control and stroke human brain. The black arrow represents changes in immunoreactivity from the respective group. Hematoxylin counterstain used for visualization of neuron nucleus (magnification 400X; Scale Bar - 20µm). (E) Triple immunofluorescence labeling for LC-3a/b (green), Sirt1 (red), and DAPI (blue) in control and stroke human brains. The representative image shows the number of LC-3a/b positive cells that co-localized with Sirt1 in stroke brain compared to control (White arrows; magnification 200X; Scale Bar - 50µm).

4. Discussion:

In the present study, we demonstrated that activation of Sirt1 induced autophagy was associated with apoptotic cell death and astrogliosis during the chronic phase of ischemic injury and treatment with curcumin + EX-527 reduced Sirt1 induced autophagy, caspase-3 mediated apoptotic cell death and astrogliosis. In humans, we observed an increase in Sirt1 induced autophagy mechanism after stroke.

The mammalian Sirt1 is highly expressed in the adult brain and plays a vital role in neuronal development, plasticity, and metabolism (Li et al. 2013), (Ramadori et al. 2008a), (Nemoto, Fergusson, and Finkel 2005). Activation of Sirt1 has been proven to aid in neuronal survival during the acute phase of cerebral ischemia in experimental animal models (Miao et al. 2016), (Teertam, Jha, and Prakash Babu 2019), (Lv et al. 2015). In recent years, emerging pieces of evidence have been linked to Sirt1 activation in the induction of autophagy, and Sirt1 can induce autophagy mechanism by direct deacetylation of various autophagy mediators, including Atg5, Atg7, and LC3 (Ren et al. 2019). Nicotinamide phosphoribosyltransferase (NAMPT), the rate limiting enzyme in NAD biosynthesis, protects against ischemia via autophagy induction in Sirt1 dependent manner and also shown that autophagy induction seems to be neuroprotective during the early phase of reperfusion, but might be harmful if it is persistently activated by ischemia (Wang et al. 2012).

The functional role of autophagy after cerebral ischemia is controversial. Some findings demonstrated that autophagy promotes ischemic pathogenesis, and other results supported its neuroprotective role against ischemia (Liu et al. 2018), (Xin et al. 2017), (Peng Wang et al. 2014), (Feng et al. 2017), (Ni et al. 2018). As part of the early protective response, a basal autophagy mechanism may promote cell survival. However, defective/excessive autophagy mechanism may trigger neuronal cell death following ischemia (Shi et al. 2012). More importantly, the defects in the transport mechanism may also lead to the accumulation of autophagosomes. Typically, autophagosome formation occurs at the axon terminal and transport retrograde to the cell body via an energy-dependent transport mechanism. During ischemic stress, defects in this retrograde transport of mature autophagosomes may lead to the accumulation of autophagosomes (Boland et al. 2008), (Hou et al. 2019). We observed impaired autophagy mechanism during the acute phase of ischemia, which may lead to the accumulation of autophagosomes and an increased LC3-II/LC3-I ratio. Importantly, we observed decrease in autophagy mechanism and reduced infarct on day7 post-stroke. Our findings on infarct volume were in line with previous observations, where infarct was shown to be reduced on day7 post-stroke (Popp et al. 2009). The alteration in the autophagy-lysosomal pathway is implicated in the manifestation of various neurodegenerative disorders in human (Nixon and Yang 2011), (Martinez-Vicente et al. 2010), (Elrick et al. 2012). Our earlier unpublished data indicate an increase in Sirt1 expression in the human brain after stroke. The present study shows that up-regulation of Sirt1 and autophagy mechanism suggest that autophagy activation may acts as a rescue mechanism in human after stroke.

Dietary polyphenol, curcumin mediated neuroprotection is by activation of Sirt1 and attenuation of caspase-3 mediated apoptotic neuronal cell death during the acute phase of ischemia (Miao et al. 2016), (W. Li et al. 2017). Further, the modulation of the autophagy mechanism is integral to protection by curcumin following I/R injury (Huang et al. 2018). On the other hand, curcumin aggravates CNS disease manifestation in the experimental lupus brain via increased immune complex deposits and enhanced inflammation (Foxley et al. 2013). Curcumin treatment was shown to have cytotoxicity against transformed neuroglial cell proliferation and growth in a dose-dependent manner (Ambegaokar et al. 2003). Our present observations might be consistent with this broader observation. In our study, curcumin treatment on day7 promoted Sirt1 induced autophagy and infarct, while treatment with curcumin + EX-527 decreased Sirt1 induced autophagy and infarct.

Both autophagy and apoptosis can act cooperatively to induce neuronal cell death. Autophagy can act as an antagonist or promoter of apoptosis after ischemia-reperfusion (Xing et al. 2012), (Pei Wang et al. 2014). Beclin-1 serves as a crossroads to lead neuronal cells to autophagy or apoptosis. The anti-apoptotic BCL-2 can directly bind to Beclin-1 and promotes activation of apoptotic cell death, and disruption of BCL-2-Beclin-1 interaction promotes autophagy mechanism (Anon n.d.). The protective effect of rapamycin following cerebral ischemia is by activation of the autophagy mechanism and inhibition of mTOR mediated apoptotic signaling pathway (Carloni et al. 2010), (Nazarinia et al. 2019). Caspases can convert various autophagy mediators to pro-apoptotic proteins to trigger apoptotic cell death. Caspase-3 is the one of the principle effector caspase, whose role in autophagy regulation has been studied most. Caspase-3 can promote or inhibit autophagy mechanism by directly interacting with various autophagy mediators including Beclin-1, Atg-4d, and Atg-5 (Tsapras and Nezis 2017). Interestingly, we observed increased caspase-3 immunoreactivity and Sirt1 mediated autophagy upon curcumin treatment on day7 and curcumin + EX-527 treatment decreased caspase-3 immunoreactivity and autophagy mechanism on day7 after stroke.

In response to central nervous system injury, astrocytes undergo dramatic changes in morphology, and they become reactive. This process is known as reactive astrogliosis. The increase in GFAP expression is the most important marker for reactive astrocytes in the central nervous system injuries (Sofroniew 2009). The functional role of the typical glial scar is controversial. It can prevent axonal sprouting, thereby prevents recovery. However, the glial scar can also act as a barrier to restrict the infiltration of inflammatory cells and infectious agents from the injured necrotic region during the chronic phase of ischemia. Recent observations report that astrogliosis/scar formation along the border of the infarct starts from about 7-10 days after ischemia and persists for the lifetime of the stroke patient (Sofroniew and Vinters 2010). We observed increased reactive astrogliosis on day7 and in curcumin treated group after day7 post-stroke. Interestingly treatment with curcumin + EX-527 reduced reactive astrogliosis on day7 after stroke.

If Sirt1 mediated autophagy mechanism plays a biphasic role after stroke, caution is needed for targeting Sirt1 induced autophagy mechanism. However, the ischemic zone may be a dynamic and flexible area during the chronic phase of ischemia, and the biphasic role of Sirt1 mediated autophagy may be due to the involvement of multiple signaling pathways of the neurovascular unit. We need careful characterization of the curcumin dosage and the molecular mechanisms associated with the transition from acute recovery to delayed injury. More importantly, astrogliosis/scar is a typical protective/detrimental response initiated by reactive astrocytes during the chronic phase of ischemia to prevent damage/repair. Curcumin treatment on day7 promoted scar formation and partial inhibition of Sirt1 expression with EX-527 in combination with curcumin decreased scar formation. Here activation of Sirt1 induced autophagy after curcumin treatment might interfere with the endogenous repair mechanisms by speeding up the scar formation and promote more damage. Although the significant limitation of this study is that we assessed the effect of curcumin on Sirt1 induced autophagy at a single time point with a single dose of curcumin on day7 after ischemia since the neuroprotective role of curcumin is well established in different settings. However, we need in-depth experimental approaches to characterize the role of curcumin and astrogliosis during the chronic phase of ischemia. Our study concludes that curcumin treatment during day7 after ischemia promoted infarct and astrogliosis via Sirt1 induced autophagy. Sirt1 mediated autophagy may interfere with the post-stroke recovery by promoting scar formation during the chronic phase of ischemia in the brains of experimental rat. In humans, activation of Sirt1 induced autophagy may act as a pro-survival response to ischemic stress and we need further experimental approaches to understand the role of Sirt1 induced autophagy in humans after stroke.

Chapter-3

Up-regulation of Sirt1/miR-149-5p signaling may play a role in resveratrol induced protection against ischemia via p53 in rat brain.

1. *Introduction:*

Stroke, a severe threat to the global population with a high incidence, high disability, and high mortality (Benjamin et al. 2017). The incidence of stroke will also increase as population ages. Stroke can be either ischemic or hemorrhagic. Ischemic stroke is the dominant subtype, which accounts for approximately 87% of all stroke cases (Amarenco et al. 2009), (Shiber et al. 2010a). Currently, tissue plasminogen activator (tPA) and thromboembolism are the best therapeutic interventions for acute ischemic stroke within the narrow window period. These treatments can lead to reperfusion-induced secondary injuries, including inflammation, apoptosis, and increased production of oxidative agents (Tobin et al. 2014), which may lead to blood-brain barrier breakdown and aggravated brain damage. However, the ineffectiveness of existing therapies shows that there should be a need for alternative therapeutic strategies.

Sirt1 is evolutionarily well conserved, class-III nicotinamide adenine dinucleotide (NAD) dependent deacetylase and the best-characterized Sirtuin among the seven Sirtuins(Shin-ichiro Imai et al. 2000). Sirt1 is predominantly expressed in the brain (Ramadori et al. 2008b). Several studies reported that Sirt1 is an essential regulator of neuronal differentiation, synaptic plasticity, longevity (Hisahara et al. 2008b), (Michan et al. 2010), (Cohen et al. 2004) and various stress-responsive transcription factors including p53 that mediates inflammation, and apoptosis (Yeung et al. 2004b), (Vaziri et al. 2001b), (Motta et al. 2004).

The transcription factor p53 is the genotoxic stress activator, which regulates the cell cycle, apoptosis, and DNA repair (Vousden and Lu 2002b), (Guo et al. 2015b). Increasing evidence demonstrated that p53 contributes to neuronal apoptosis via mitochondrial-dependent intrinsic pathway and activation of caspase-3 during ischemia (Chipuk et al. 2004), (Liu et al. 2019). Pharmacological inhibition of p53 has shown to be neuroprotective by downregulation of apoptotic mediators in various stroke models (Crumrine et al. 1994), (LEKER et al. 2004), (Cheng et al. 2003), (Nijboer et al. 2011). Recent findings support that p53 can also act as a transcription factor to modulate the function and expression of multiple miRNAs (Corney et al. 2007). On the other hand, numerous microRNAs have been involved in the regulation of p53 expression and function by targeting the p53 mRNA (Le et al. 2009), (Hu et al. 2010).

MicroRNAs are evolutionarily conserved, single-stranded noncoding RNAs with 18-25 nucleotides. They modulate protein expression by binding to complementary 3’UTR of target mRNA, leading to their translational inhibition (Friedman et al. 2009), (Djuranovic, Nahvi, and

Green 2012). Increasing evidence has shown that miRNAs are known to play an essential role in various pathological processes, including tumorigenesis, inflammation, neuronal plasticity, and neurodegeneration (Mocellin, Pasquali, and Pilati 2009), (Su, Aloi, and Garden 2016), (AUMILLER and FORSTEMANN 2008), (Bushati and Cohen 2008). miR-149 is found to be dysregulated in multiple tumor conditions (Ow, Chua, and Bay 2018), and the precise role of miR-149 after ischemia remains unclear. We predicted miR-149 targets with the help of Target Scan and found that miR-149-5p likely regulate p53 gene expression and p53 is one of the possible targets of miR-149-5p after I/R injury.

In this context, we emphasized the role of Sirt1/p53/miR-149-5p signaling and its modulation by resveratrol during ischemic pathogenesis in the rat model. We also established whether miR-149-5p in regulating neuronal cell apoptosis through Sirt1/p53 axis after stroke.

2. Materials & Methods:

2.1 Transient Middle Cerebral Artery Occlusion (MCAO):

Animal experiments were performed on male Sprague-Dawley (SD) rats (250-300 gr). All the experimental approaches were carried out following the Institutional and National Animal Ethical Committee (IAEC). Rats were procured from the National Centre for Laboratory Animal Sciences (NCLAS), Hyderabad, India. Animals were housed under controlled environment with free access to food and water. Resveratrol is purchased from Sigma (USA). The rats were randomly divided into three groups: (i) Sham group, (ii) MCAO group, (iii) MCAO + Resveratrol group (dissolved in DMSO + 0.9% NaCl; 20mg/kg through IP route, prior 30 min of MCAO). Focal cerebral ischemia was induced by the occlusion of a middle cerebral artery (MCA) as previously reported (Longa et al. 1989b). Rats were anesthetized with an IP injection of Ketamine (60mg/Kg) and Xylazine (10mg/kg). The left common carotid artery (CCA), internal carotid artery (ICA), and the external carotid artery (ECA) were identified through a middle neck incision. Both ECA and CCA were ligated distally. A 3-0 nylon monofilament was gently advanced from ECA by arteriectomy, into ICA to block the origin of the left MCA. For the sham-operated group, monofilament was advanced till the junction of ICA from CCA, but MCA was not occluded. After 60min of occlusion, the monofilament was carefully removed and allowed for reperfusion; the neck incision was closed, and animals were allowed to recover from the sedation.

2.2 Neurological deficit score:

The neurological deficit was measured by using the Bederson scale (Bederson et al. 1986). Rats with no abnormality were scored as grade-0, rats which failed to extend forelimb contralateral to infarct were scored as grade-1, rats which shows infrequent circling towards the infarct side with decreased resistance to lateral push were scored as grade-2, rats which show hemiparesis were scored as grade-3 and rats which died due to severe lesion were scored as grade-4.

2.3 Immunoblotting:

Animals were decapitated with the overdose of pentobarbital at 24 h after reperfusion; ipsilateral brains were excised and used for further analysis. Brain samples were homogenised in RIPA buffer (150mM NaCl, 2mM EDTA, 50mM Tris-HCl (pH-7.4), 10%Glycerol, 1%NP40, and 0.5%Sodium deoxycholate). Protein quantity in the supernatant was determined by the Bradford method following centrifuging tissue homogenates at 10,000 rpm for 20 min. Total protein extract was separated by 10% SDS-PAGE gels and transferred on to nitrocellulose membrane. The membranes were incubated with 5% skimmed milk powder in Tris-buffered saline (TBS) containing Tween-20 for 90 min. Blots were incubated overnight at 4°C with primary antibody (1:1000 dilution) against Sirt1, beta-actin (cell signaling technology, USA), and p53 (Merck, USA). After washing with TBS-T, membranes were incubated with HRP labeled secondary antibody (CST, USA) specific for mouse and rabbit for 90 min at 37°C (1:3000 dilution). Immunoreactivity was visualized by using the ECL detection system (Bio-Rad, USA).

2.4 Immunohistochemistry:

Brains were fixed by trans cardiac perfusion with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA), then embedded in paraffin and sectioned into 5m thick slices using microtome (Leica, Germany). Brains were immunostained using immunohistochemistry detection kit (Bio SB, USA) against Sirt1, p53, and caspase-3. Sections were deparaffinized in three changes of xylene each for 5 min, followed by hydration in decreased alcohol gradient for each 5 min. Endogenous peroxidase was blocked by incubating slides in 3% Hydrogen Peroxide in methanol, followed by heat-induced antigen retrieval in TRIS-EDTA buffer. Non-specific binding was minimized by blocking sections with 0.25% BSA for 30 min; then brain slices were incubated with primary antibody (1:100 dilution) for 2 h 30 min at room temperature (RT), followed by incubation with secondary antibody for 45 min at RT. Staining was visualized by developing the sections in buffer containing 3’-3’ diaminobenzidine (DAB) (supplied in kit) for 5 min. The sections were counterstained with Hematoxylin for 5 min. Finally, dehydrated and mounted with coverslips. Sections were photographed under the light microscope (Olympus, Japan) with 400 X magnification.

2.5 Infarct volume measurement:

Brains were frozen immediately at -20° C for 30 min after excision from rats after 24 h following MCAO. Frozen brains were cut into 2mm thick coronal slices and stained with 2% 2,3,5- triphenyl tetrazolium chloride (TTC) (Sigma-Aldrich, US) at 37° C for 30 min, followed by 4% paraformaldehyde (PFA) fixation. Infarct area from all coronal slices was multiplied by the thickness of the brain section to get total infarct volume. Sections were photographed and analyzed with ImageJ software. (NIH, US).

2.6 Hematoxylin & Eosin staining:

Paraffin-embedded brain sections were dewaxed in xylene and then hydrated in a decreased gradient of alcohol. Brain sections were stained with Hematoxylin and Eosin, followed by dehydration in increased alcohol gradient. Sections were mounted with DPX. Images were captured under the light microscope with 400 X magnification.

2.7 Caspase activity assay:

Total protein lysates (100g) from control and experimental rat ipsilateral brains were homogenized in RIPA and dissolved in 100 ml of analysis buffer (100mM NaCl, 10mM DTT, 20mM HEPES pH 7.4, 10%sucrose, 0.1% CHAPS, 1mM EDTA) and incubated at 37° C for 6 h. After incubation 5 ml of Caspase Substrate N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-tri fluoromethylcoumarin (Ac-DEVD-AFC, Sigma, US) was added to protein lysate and make up the final concentration of 1ml with analysis buffer, followed by incubation for 1 h at 37° C. Fluorescence emission during the cleavage of AFC substrate was measured by a fluorescence spectrophotometer (FluoroMax) at excitation 400 nm and emission 450–505nm. The fluorescence intensity of the cleaved AFC substrate is directly relating to the cumulative activity of effector caspases 3,6 and 7.

2.8 Quantitative Real-Time PCR (q-RT-PCR):

Total RNA was extracted by using TRizol (Sigma, US) reagent according to the manufacturer's protocol and quantified with NanoDrop spectrophotometer (Thermo Scientific, US). PCR reactions were performed with SYBR Ex Taq (Takara, Japan) in 7500 fast Real-time PCR system (Applied Biosystems, US). For miRNA expression RNA was reverse transcribed using miRNA first strand synthesis kit (Takara, Japan) with universal reverse primer according to manufacturer's instructions. Relative fold change in miR-149-5p expression was normalized to U6 small nuclear RNA (supplied in kit) as an internal control. The primer sequence for mature miR-149-5p is – 5 – TCTGGCTCCGTGTCTTCACTCC–3'.

2.9 Network construction and analysis:

Disease query in STRING(Szklarczyk et al. 2011) database, which is a Cytoscape (V.3.7.1)(Shannon et al. 2003) plug-in was used for disease query with keyword "Stroke," and the highest confidence score of 1.0 was chosen to get the most accurate interactions with 1000 nodes output. STRING disease query creates network using gene list generated by database named DISEASES(Pletscher-Frankild et al. 2015), which is a web resource that integrates evidence on disease-gene associations from automatic text mining, manually curated literature, and genome-wide association studies providing an updated disease associated gene list. Degree filter was used to create the network in which all the nodes have a minimum of 4 interactions with other nodes and finally TP-53 interacting gene network was isolated from the filtered network with Cytoscape node selection functionality. Functional and pathway enrichment analysis: The list of TP-53 interacting gene identifiers generated from Cytoscape was uploaded in ClueGo(Bindea et al. 2009) plugin for Cytoscape to create functional and pathway analysis. Two-sided tests based on the hypergeometric distribution was used to generate P-value followed by the multiple test correction [Benjamini-Hochberg adjustment(Thissen, Steinberg, and Kuang 2002). P-value <0.05 and Kappa score threshold (≥0.4) was used to generate the pathway network which was divided into functional groups, in which a node represented a pathway, and an edge between two nodes indicated that the two pathways shared common genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) was selected in ClueGo settings for pathway network generation. Further pathways were deleted manually to trim the network to only pathways related to stroke and gene were mapped to generate a network showing common genes between pathways using mapping functionality provided in ClueGo software. Finally, activation inhibition network was curated by manually selected genes overlapping between different pathways using CluePedia knowledgebase in ClueGo.

3. Results:

3.1 Expression of miR-149-5p in rat cerebral tissue following MCAO:

To investigate the role of miR-149-5p in the regulation of ischemic neuronal death, we first assessed the miR-149-5p expression levels in rat brain after stroke, using q-RT-PCR. We found that miR-149-5p levels were significantly decreased in the post-stroke group compared with the sham control ($p<0.001$, Figure. 18). This data suggests that downregulation of miR-149-5p may play an essential role in the ischemic cell death.

3.2 Resveratrol treatment upregulates miR-149-5p via Sirt1 activation:

We further examined the correlation between Sirt1 and miR-149-5p during ischemia. Immunoblotting demonstrated that expression of known anti-apoptotic Sirt1 activity was substantially decreased in the MCAO group compared to the sham control ($p<0.01$, Figure. 21 A). Treatment with resveratrol significantly increased the activity of Sirt1 ($p<0.01$, Figure. 21 A) and upregulated the expression of miR-149-5p ($p<0.001$, Figure. 18). These observations confirmed that neuroprotection conferred by Sirt1 maybe in association with the upregulation of miR-149-5p.

3.3 Sirt1/miR-149-5p axis confers functional protection against I/R by targeting p53:

Next, we explored the role of Sirt1/miR-149-5p axis on p53 transactivation. We found that p53 protein expression was upregulated following MCAO in rat cerebral tissue compared to the sham control ($p<0.001$, Figure. 21 B), which was contrary to the expression of miR-149-5p. Treatment with resveratrol significantly reduced p53 activity through Sirt1 modulation, compared to the MCAO group ($p<0.001$, Figure. 21 B).

3.4 Subcellular localization of Sirt1, p53 & caspase-3:

Immunohistochemistry was performed to identify the subcellular distribution of Sirt1, p-Jnk, & caspase-3 in brains of control and experimental rats. Sirt1 immunoreactivity was mainly found in neuron nucleus in the brains of control rat. The decrease in nuclear immunoreactivity was observed in stroke brain compared to control. More importantly, we saw Sirt1 positive cells in both the nucleus and cytoplasm in the brains of the ischemic rat. The number of Sirt1 positive cells was increased on resveratrol treatment compared to the MCAO group (Figure. 22 A). No p53 immunoreactive cells were found in the brains of control rat. A significant increase in p53 immunoreactive cells was observed in both nucleus and cytoplasm following I/R. Resveratrol treatment has shown a few p53 positive cells compared to control (Figure. 22 B). A few numbers of caspase-3 neuron cytoplasm and cell processes were identified in control. Caspase-3 immunoreactivity was observed in both neuron nuclei and cytoplasm in brains of the experimental rat (Figure. 22 C).

3.5 p53 downregulation decreases caspase-3 via Sirt1/miR-149-5p axis:

Further, we sought to determine the role of caspase-3, one of the effector caspases, which is activated by p53 dependent intrinsic apoptotic mechanism. We observed an increase in caspase-3 immunoreactivity in the neuron nuclei following I/R injury compared with the sham control. In control brain, a few caspase-3 positive cells were limited to neuron cytoplasm. A few caspase-3 immunoreactive nuclei were observed in brains on resveratrol treatment compared to the MCAO group (Figure. 22 C).

3.6 Sirt1/miR-149-5p axis decreases effector caspase activity:

The brains of ischemic rats have shown high effector caspase activity compared to the brains of control rat ($p<0.03$). Activation of Sirt1/miR-149-5p axis significantly decreased effector caspase activity ($p<0.03$) compared to stroke brains (Figure. 23).

3.7 Sirt1/miR-149-5p axis decreases infarct and neuronal cell death:

Hematoxylin & Eosin staining revealed cortical pyramidal neuronal cell loss in brains of experimental rat compared to brains of control rat. Sirt1 activation with resveratrol decreased pyramidal neuronal loss compared to brains of the experimental rat (Figure. 20). Moreover, brains of resveratrol treatment showed a substantial decrease in infarct compared to brains of the experimental rat ($p<0.01$, Figure. 19).

3.8 Network analysis & pathway enrichment:

Stroke network comprised a total of 965 genes with 8137 edges, which includes 13 miRNA genes (miR-149-5p, 9-5p, 499a-5p, 29b-3p, 223-3p, 210-3p, 155-5p, 146a-5p, 145-5p, 126-3p, 124-3p, let-7f-5p), was constructed with the help of STRING, which supports the involvement of miR-149-

5p after I/R injury (Figure. 24). Next, we filtered important hubs to minimum four interacting partners, using Cytoscape 4degree filter, which resulted in a network of 365 nodes with 3528 interactions (Figure. 26 A). Genes with higher degree indicate a higher number of possible interactions with other genes and more likely to involve in stroke pathogenesis (Table. 2). The p53 gene network was constructed with 49 genes, which shown to have direct interaction with p53 from the filtered network (Figure. 26 B). The p53 pathway network was constructed (Figure. 25), trimmed further and aligned manually based on the functional importance of pathways in neuronal death and size of the functional modules (Figure. 26 C & D) (Table. 3). Finally, p53 activation/inhibition network was constructed to emphasize the interaction between p53 and its interacting partners (Figure. 26 E & F). We found that 15 genes were directly interacting and activating p53 includes (BAX, EP-300, MAPK8, 3, 10, 9, 14, & 1, NGFR, TRAF6, HDAC9, BDNF, PARP, GSK3B, & SIRT1), and 5 genes were directly interacting and inhibiting p53 activity includes (SIRT1, FOS, AKT1, JUN, & EP300). We found that 12 genes were directly interacting and activated by p53 includes (SIRT1, BAX, PTGS2, MAPK1, 3, 10, PTEN, CASP8, VEGFA, HGF, & SERPINE-1.

Figures:

Figure. 18:

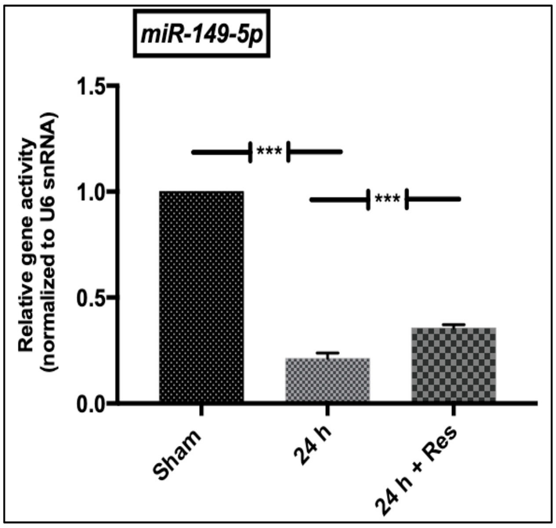


Fig. 18 Characterization of miR-149-5p expression in rat cerebral tissue following MCAO. The RT-PCR results showed that relative expression of miR-149-5p was down-regulated in ischemic rat brain after 24 h compared to sham. Resveratrol treatment significantly upregulated the expression of miR-149-5p compared to MCAO group. U6snRNA was used as an internal reference. The 2ΔΔCt method used to determine relative fold change in gene expression. Sham, control group; 24 h, ischemic group; 24 h Res, resveratrol group; Data represented as mean ± SE. *** p<o.oo1 sham versus 24 h; *** p<o.oo1 24 h vs. 24 h Res.

Figure. 19:

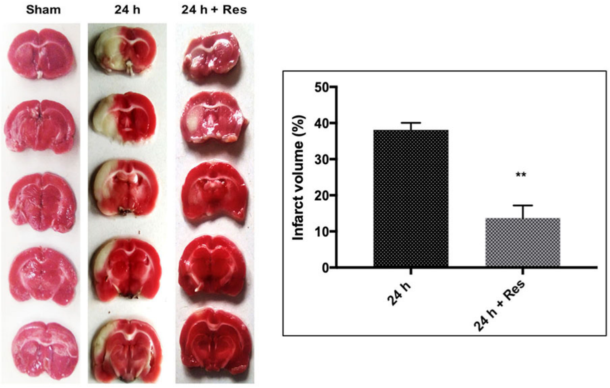


Fig. 19 Sirt1 decreases infarct following MCAO. Representative images of brains from control, stroke, and resveratrol treatment after stained with TTC. The graph showed that the infarct was substantially decreased in brains of resveratrol treatment compared to brains of 24 h. Sham, control group; 24 h, ischemic group; 24 h Res, resveratrol group; The data was subjected to paired t-test and represented as percentage of infarcted tissue. *p<o.o5 24 h vs. 24 h Res.

Figure. 20:

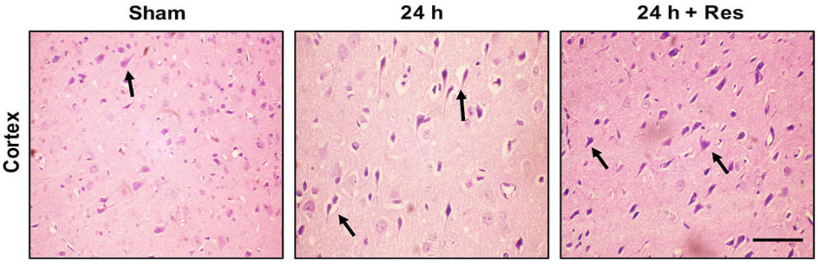


Fig. 20 Sirt1 attenuates neurodegeneration following MCAO. Representative H&E images of brains from control, stroke, and resveratrol treatment. Neuronal loss was decreased in brains of resveratrol treatment compared to brains of 24 h. The morphological changes in cortical pyramidal neurons were indicated in black arrow. Sham, control group; 24 h, ischemic group; 24 h Res, resveratrol group (Scale Bar - 50µm; magnification - 400 X).

Figure. 21:

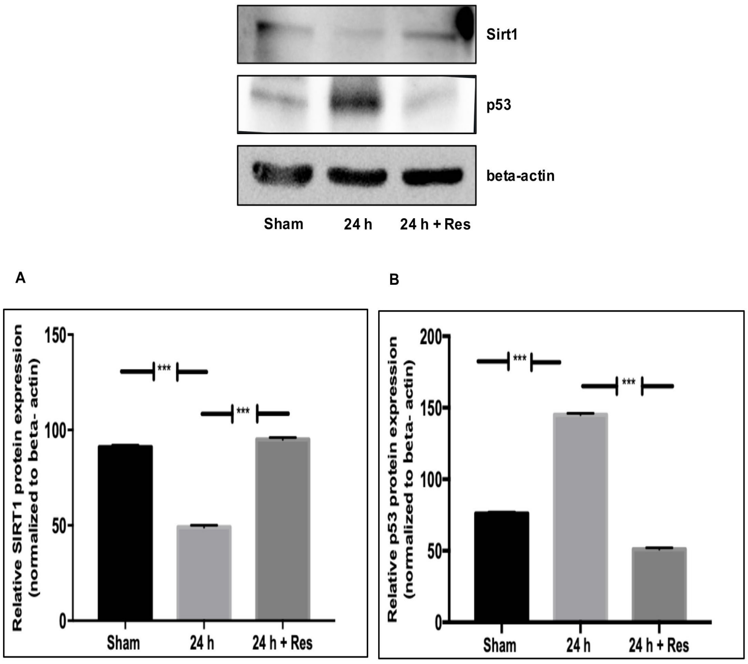


Fig. 21 The effect of resveratrol on Sirt1, & p53 protein expression following MCAO. The Immunoblot images of Sirt1 (A) and p53 (B) is shown in the figure. Resveratrol treatment up-regulated Sirt1 functional activity and decreased p53 expression. Actin is used as an internal control. Sham, control group; 24 h, ischemic group; 24 h Res, resveratrol group; Data represented as mean ± SD. $*p<0.05$ 24 h versus 24 h Res.

Figure. 22:

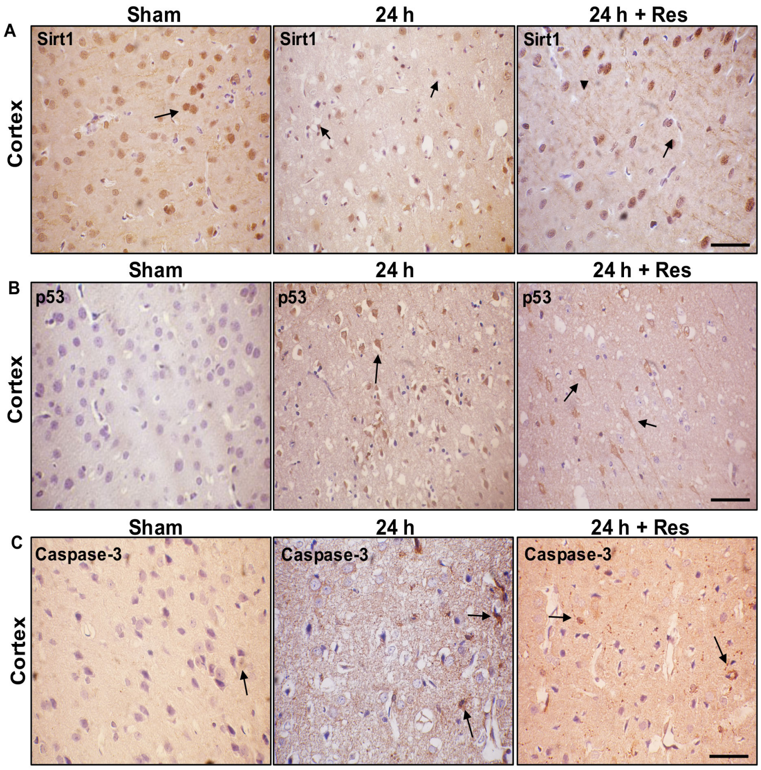


Fig. 22 Subcellular localization of Sirt1, p53, & caspase-3 in brains of control and experimental rats. Sirt1 immunoreactivity in brains of experimental rat is characterized by week nuclear immunoreactivity compared to control while the number of Sirt1 positive cells were increased on resveratrol treatment compared to brains of 24 h (A). The number of p53 and caspase-3 positive cells was found to be decreased in brains of resveratrol treatment compared to brains of 24 h (B & C). Black arrows represent the changes in Sirt1, p53 & caspas-3 immunoreactivity. Sham, control group; 24 h, ischemic group; 24 h Res, resveratrol group (Scale Bar - 50µm; magnification - 400 X).

Figure. 23:

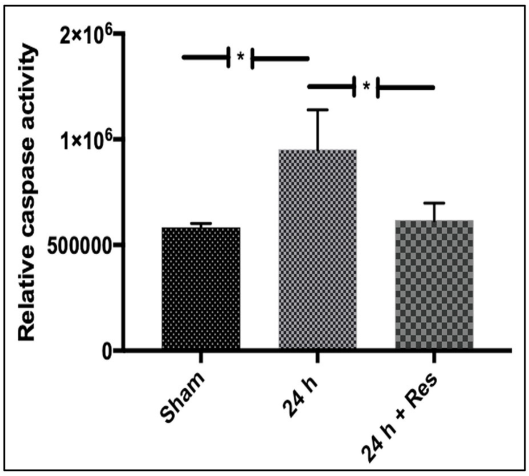


Fig. 23 Sirt1 decreases effector caspase activity. The graph shows the expression of effector caspases (caspase-3, 6, & 7) in brains of control, stroke, and resveratrol treatment. Resveratrol treatment leads to a substantial decrease in effector caspase activity compared to the brains of 24 h. Sham, control group; 24 h, ischemic group; 24 h Res, resveratrol group. $*p<0.05$ 24 h versus 24 h Res.

Figure. 24:

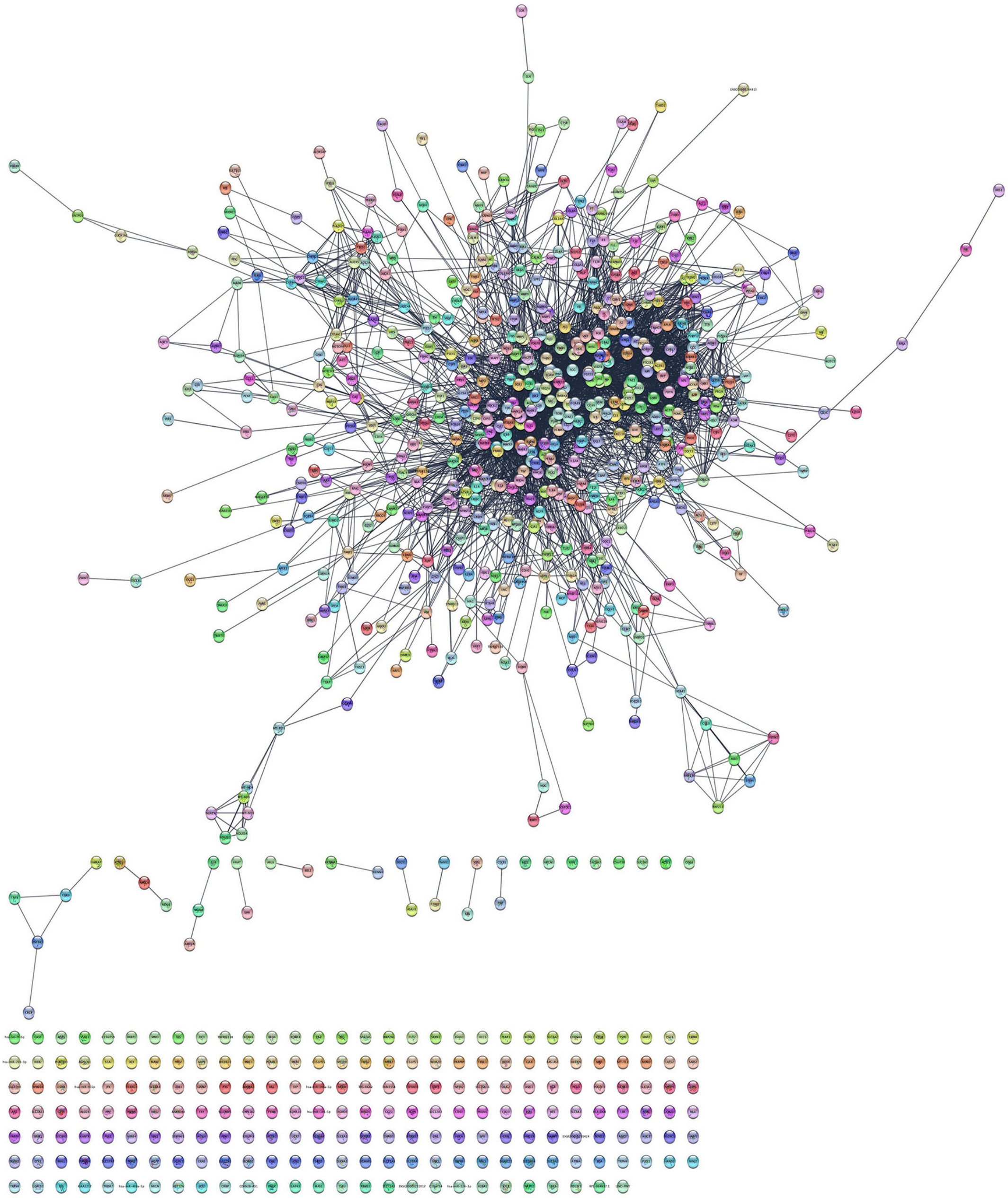


Fig. 24 Stroke network construction using STRING. The complete network of stroke was constructed by using Cytoscape plug-in STRING database with key terms "stroke" using gene list generated by database named DISEASES.

Figure. 25:

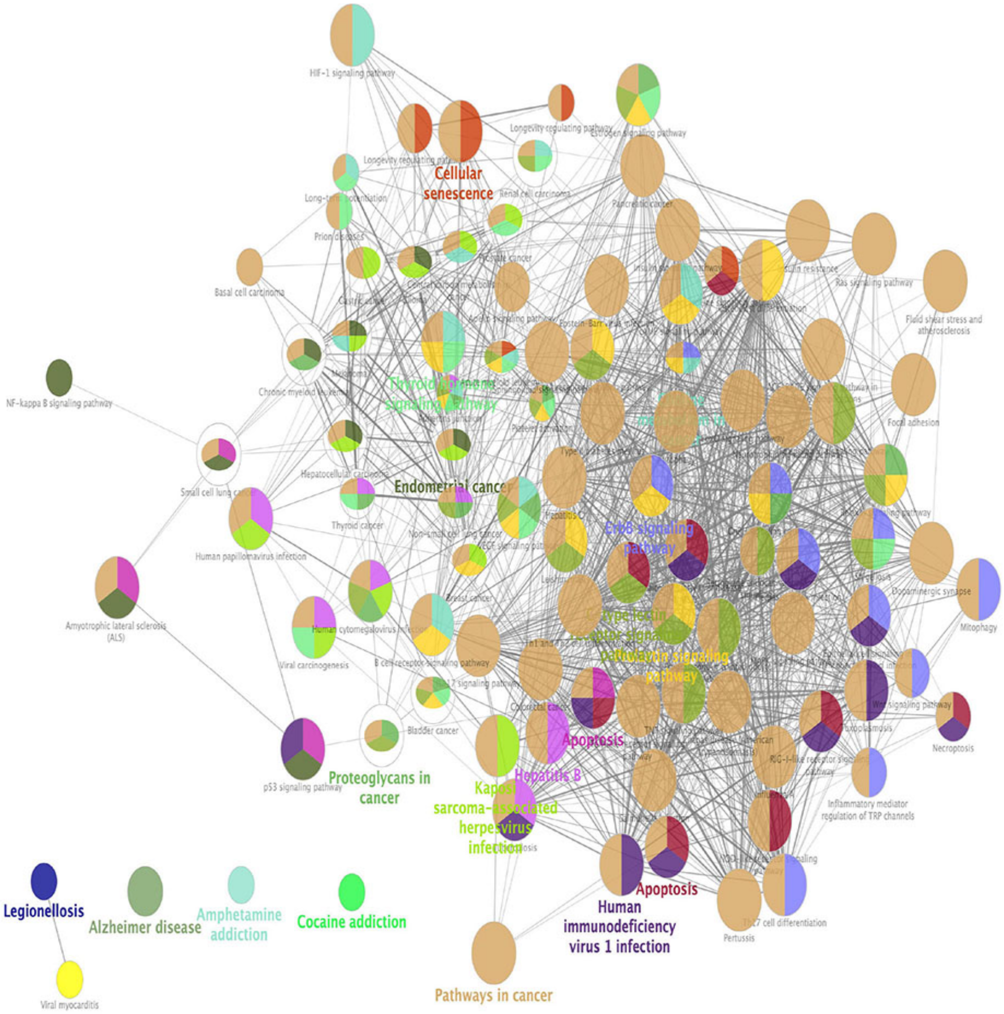


Fig. 25 Functionally grouped network for the TP53 and its interacting genes. Functionally grouped network with terms as nodes linked using ClueGO analysis, where only the label of the most significant KEGG pathway term per group is shown. The node size represents the term enrichment significance and functionally related groups partially overlap. Edges between nodes suggest there are common genes among the pathways. Two-sided tests based on the hypergeometric distribution was used to generate P-value followed by the multiple test correction [Benjamini-Hochberg adjustment, respectively P-value <0.05 and Kappa score threshold ≥0.4 were used to create the pathway network.

Figure. 26:

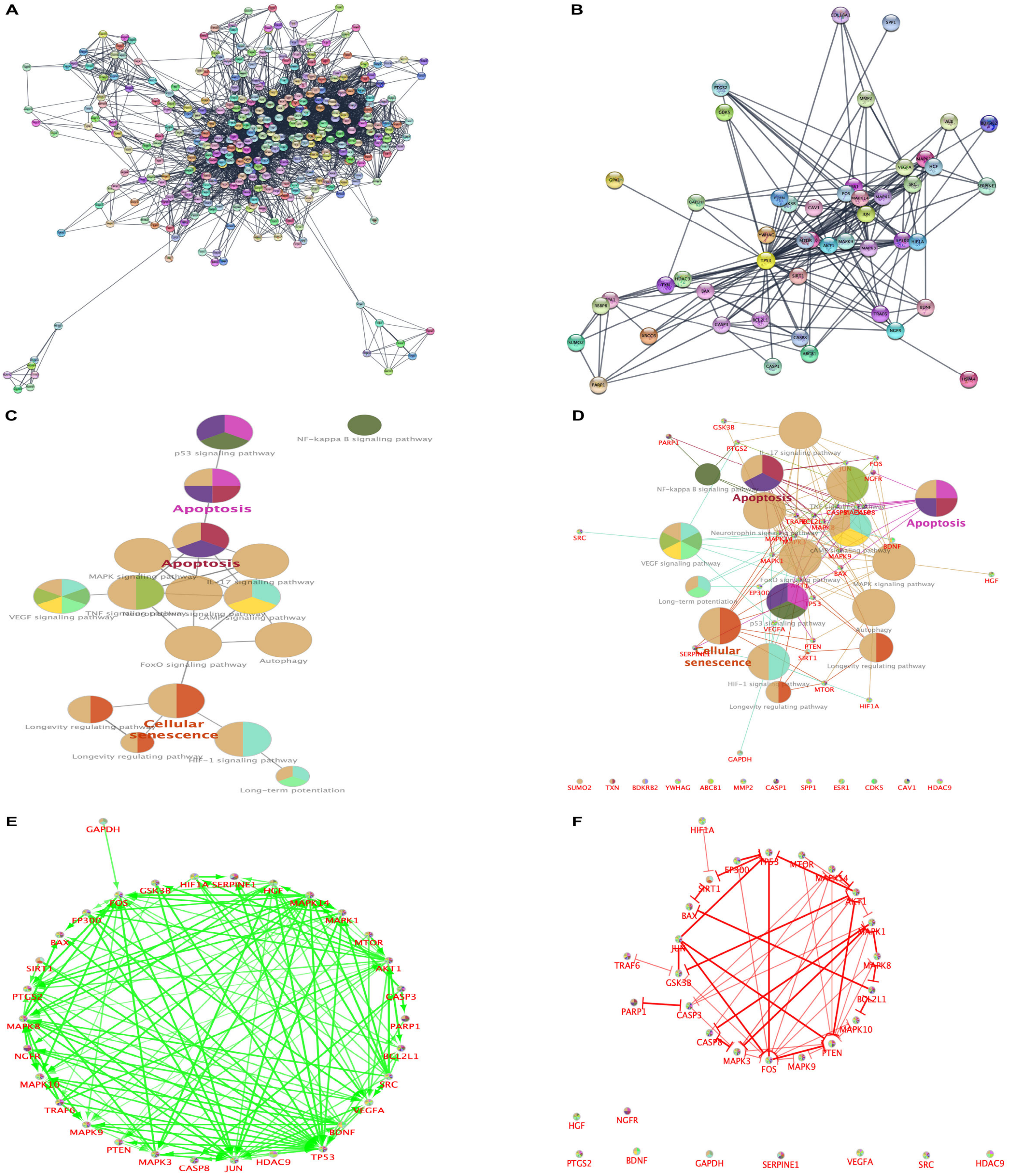


Fig. 26 Stroke network and pathway enrichment. (A), represents the network of important genes in stroke as each gene has a minimum of four interacting genes. (B), the network of TP 53 interacting genes curated from complete Stroke network from (A). The highest confidence score of 1.0 was used to create the network. (C), Curated pathway module network from complete pathway network created for TP 53 and its interacting genes. (D), the network represents the mapped genes on the curated pathway network, where the smaller nodes represent genes and larger nodes represent pathways. Genes without edges have no mapped pathways. (E), activation network of genes associated in curated pathway network, where the direction of the arrow shows the gene is activated, the bi-directional arrow shows both genes are activating each other. (F), Suppression network of genes associated in curated pathway network, the bar at the end of represent gene being repressed, genes without any edge represent no suppression relation with other genes.

Table. 2:

Important hubs from Filtered stroke network		
S.No	Gene name	Degree
1	APP	101
2	KNG-1	89
3	GNB-3	73
4	JUN	73
5	VEGFA	67
6	AGT	66
7	MAPK-1	65
8	EGF	64
9	F-2	64
10	IL-8	60

Table. 2 Important gene hubs from Filtered stroke network. Summary of important gene hubs from a 4-degree filtered stroke network. The highest number of degree indicates the highest number of possible interactions with other genes within the network.

Table. 3:

P53 pathway Network		
S.No	GO term	Associated Genes found
1	Apoptosis pathway	AKT1, BAX, BCL2L1, CASP-3, 8, FOS, JUN, MAPK1, 10, 3, 8, 9, PARP & TP53, NGFR
2	IL-17 pathway	FOS, CASP-3, 7 & 8, GSK3B, JUN, MAPK1, 10, 14, 8, 9, & 3, PTGS2, TRAF6
3	Long term potentiation pathway	EP-300, MAPK1, MAPK3
4	Neurotrophin pathway	BAX, BDNF, GSK3B,JUN, MAPK1 ,10, 14, 3, 8, 9, AKT1, NGFR,TP53, TRAF6
5	cAMP pathway	BDNF, EP-300, AKT1, FOS, JUN, MAPK1, 10, 3, 8 & 9
6	P53 pathway	BAX, BCL2L1,CASP3, 8, PTEN, SERPINE1, TP53
7	NF-Kb pathway	BCL2L1, PARP, PTGS2, TRAF6
8	VEGF pathway	MAPK1, 14, 3, PTGS2, SRC1, AKT1, VEGFA
9	TNF pathway	CASP3, 8, FOS, JUN, AKT1, MAPK1,10 ,14, 3, 8, 9, PTGS2
10	MAPK pathway	BDNF, CASP3, FOS, HGF, JUN, MAPK1, 10, 14, 3, 8, 9, NGFR, TP53, AKT1, TRAF6, VEGFA
11	Longevity pathway	BAX, MTOR, SIRT1, TP53, AKT1
12	FOXO pathway	EP-300, AKT1, MAPK1, 10, 3, 14, 8 & 9, PTEN, SIRT1
13	Autophagy pathway	HIF-1A, mTOR, PTEN, AKT1, BCL2L1, MAPK1, 10, 3, 8, 9, TRAF6
14	HIF-1 signaling	EP-300,GAPDH, HIF-1A, MAPK1, 3, mTOR, SERPINE1, VEGFA
15	Cellular senescence	mTOR, PTEN, MAPK1, 14, 3, AKT1, SERPINE1, SIRT1, TP53

Table. 3 Curated p53 pathway network. The curated p53 pathway network was manually trimmed and aligned from complete p53 network, which represents mapped genes and their associated pathways, which are mainly involved in neuronal survival and neuronal cell death after ischemia.

4. Discussion: -

The significant finding of this study is that miR-149-5p was downregulated and inversely correlated with p53 expression following MCAO. We have shown that activation of Sirt1 decreased neuronal cell loss by modulating miR-149-5p/p53 axis after ischemia in the brains of the experimental rat. Our results support that miR-149-5p is involved in the regulation of caspase-3 mediated apoptotic neuronal cell death via Sirt1/p53 axis, which could be a potential therapeutic target for cerebral I/R injury.

The early phase of necrotic cell death in the ischemic core followed by reperfusion-induced secondary injury. I/R induced injury is mediated by diverse molecular mechanisms, including increased oxidative stress, excitotoxicity, and apoptosis (Iadecola and Anrather 2011), (Puyal, Ginet, and Clarke 2013). Apoptosis is the principal mediator of neuronal cell death in the peri-infarct region, and caspase-3 is the executioner caspase responsible for apoptotic cell death (Broughton et al. 2009), (Onténiente et al. 2003). Sirt1 plays a critical role in longevity and oxidative stress (Cohen et al. 2004), (Brunet et al. 2004). Sirt1 is well known for its neuroprotective credentials in various neurodegenerative disorders (Donmez 2012b). Neuroprotection conferred by Sirt1 against ischemic preconditioning and acute ischemia is well-reviewed (Hernandez-Jimenez et al. 2013), (Yan et al. 2013b). One of the survival mechanism thought to be regulated by Sirt1 is by inhibition of p53 functional activity by deacetylation of a critical lysine residue, thereby inhibits caspase-3 dependent apoptotic cascade (Qian et al. 2017), (Hu et al. 2017), (Mao et al. 2019). Activation of Sirt1 enzymatic activity by pharmacological means confers neuroprotection against acute ischemia in various experimental animal models (Raval et al. 2006), (Miao et al. 2016). Resveratrol is the potent activator of Sirt1, found abundantly in grape skin and seeds (Howitz et al. 2003). Resveratrol has been proven to reduce infarct size in various experimental animal models subjected to cerebral ischemia (Huang et al. 2001), (Raval et al. 2006), (Della-Morte et al. 2009), (Koronowski et al. 2017), (Sinha, Chaudhary, and Gupta 2002b), (Wang et al. 2002). Resveratrol induced neuroprotective properties are mainly associated with its anti-inflammatory and anti-apoptotic properties (Singh, Agrawal, and Doré 2013).

Emerging evidence has shown that p53 modulates miRNA biogenesis by associating with various miRNA processing enzymes, including RNA helicase and drosa (Suzuki et al. 2009). Recent observations found that p53 regulates the transactivation of miR-34 by directly binding to the p53 responsive elements located within the gene encoding miR- 34, thereby altering Sirt1 expression and promoting apoptosis (Chang et al. 2007), (Yamakuchi, Ferlito, and Lowenstein 2008). Further, microRNAs can silence p53 mRNA expression, thus modulates the appearance and function of Sirt1 feedback loop to acetylate the p53 and therefore decrease the caspase-3 dependent apoptotic signaling (Lou et al. 2015), (Lu and Wang 2017). Recent findings reported that microRNAs involved in the alteration of various target genes relating apoptotic neuronal death via p53 dependent and independent manner following I/R. For instance, miR-124 decreases neuronal apoptosis by downregulation of inhibitory member of the apoptosis stimulating proteins of p53 family (iASPP) in experimental stroke (Liu et al. 2013). MicroRNA Let-7 has been shown to target apoptotic caspase-3, which found to be downregulated after stroke (Tan et al. 2009), (JIN et al. 2016). MicroRNA-149 is well known for its association in tumor suppression, inflammation, and apoptosis (Bischoff et al. 2014), (Chen et al. 2018), (Lin, Lin, and Yu 2010), (Rong Tan et al. 2013). MicroRNA-149 is deregulated in the serum of stroke patients (Jeon et al. 2013). Our results show that downregulation of miR-149-5p expression following ischemia, leading to increased neuronal damage, which is reflected by upregulation of caspase-3 mediated apoptotic cell death. Further, miR-149-5p, Sirt1 expression was upregulated, and p53 expression was significantly downregulated when exposed to resveratrol. miR-149-5p acts as a bridge molecule between the Sirt1/p53 feedback loop, in which resveratrol induces Sirt1 and then miR-149-5p, which lead to the repression of p53 and caspase-3. Several studies have reported miRNAs and their target genes, but lack systematic analysis of target gene interacting pathways. Hence our study selected comprehensive bioinformatics tools like ClueGO and KEGG pathway analysis to study p53 and its activation/inhibition network following I/R injury. p53 activation/inhibition network results have shown various well -known interactions include Sirt1 (Donmez 2012b), GAPDH (Zhai et al. 2014), and likely interacting partners include SERPINE-1 and EP-300. We sought further experimental approaches to emphasize these interactions, which could help in the rich understanding of their role in ischemic pathophysiology. In conclusion microRNA-149-5p was downregulated during ischemia after 24 h reperfusion. Resveratrol treatment significantly upregulated miR-149-5p via Sirt1 dependent manner, and miR-149-5p upregulation decreased neuronal loss by likely bind to p53, thereby decreased caspase-3 activity.

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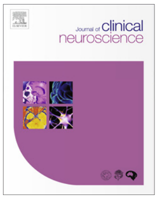

Experimental study

Up-regulation of Sirt1/miR-149-5p signaling may play a role in resveratrol induced protection against ischemia via p53 in rat brain

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ABSTRACT

Micro-RNA(miRNA) are well studied small noncoding RNA, which plays a diverse role in the regulation of vital elements in cell survival and apoptosis. However, the functional significance of miRNAs after the pathogenesis of ischemic stroke remains unclear. The present study is designed to investigate the regulatory role of miR-149-5p on Sirtuin-1/p53 axis during ischemic-reperfusion-induced injury. Middle cerebral artery occlusion (MCAO) was performed by nylon monofilament for 60 min. Resveratrol was administered via intraperitoneal (IP) route, 30 min before the MCAO. Our study demonstrated that the miR-149-5p levels were markedly decreased at 24 h after ischemic-reperfusion (I/R) injury. Further, we observed decreased p53 protein expression and increased miR-149-5p activity on sirtuin1 (Sirt1) activation with resveratrol after 24 h following MCAO. Moreover, immunohistochemistry studies found that resveratrol treatment significantly decreased the immunoreactivity of p53 and caspase-3 on activation of Sirt1/miR149-5p axis. In conclusion, our findings suggest that miR-149-5p could play a regulatory role in neuronal cell death via Sirt1/p53 axis, which offers a new target for novel therapeutic interventions during acute ischemic stroke.

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1. Introduction

Stroke, a severe threat to the global population with a high incidence, high disability, and high mortality [1]. The incidence of stroke will also increase as population ages. Stroke can be either ischemic or hemorrhagic. Ischemic stroke is the dominant subtype, which accounts for approximately 87% of all stroke cases [2,3]. Currently, tissue plasminogen activator (tPA) and thromboembolism are the best therapeutic interventions for acute ischemic stroke within the narrow window period. These treatments can lead to reperfusion-induced secondary injuries, including inflammation, apoptosis, and increased production of oxidative agents [4], which may lead to blood-brain barrier breakdown and aggravated brain damage. However, the ineffectiveness of existing therapies shows that there should be a need for alternative therapeutic strategies.

Sirt1 is evolutionarily well conserved, class-III nicotinamide adenine dinucleotide (NAD) dependent deacetylase and the best-characterized Sirtuin among the seven Sirtuins [5]. Sirt1 is predominantly expressed in the brain [6]. Several studies reported that Sirt1 is an essential regulator of neuronal differentiation, synaptic plasticity, longevity [7–9] and various stress-responsive transcription factors including p53 that mediates inflammation, and apoptosis [10–12].

The transcription factor p53 is the genotoxic stress activator, which regulates the cell cycle, apoptosis, and DNA repair [13,14]. Increasing evidence demonstrated that p53 contributes to neuronal apoptosis via mitochondrial-dependent intrinsic pathway and activation of caspase-3 during ischemia [15,16]. Pharmacological inhibition of p53 has shown to be neuroprotective by down-regulation of apoptotic mediators in various stroke models [17–20]. Recent findings support that p53 can also act as a transcription factor to modulate the function and expression of multiple miRNAs [21,22]. On the other hand, numerous microRNAs have been involved in the regulation of p53 expression and function by targeting the p53 mRNA [23,24].

MicroRNAs are evolutionarily conserved, single-stranded non-coding RNAs with 18–25 nucleotides. They modulate protein expression by binding to complementary 3′UTR of target mRNA, leading to their translational inhibition [25,26]. Increasing evidence has shown that miRNAs are known to play an essential role in various pathological processes, including tumorigenesis, inflammation, neuronal plasticity, and neurodegeneration [27–30]. miR-149 is found to be dysregulated in multiple tumor conditions [31], and the precise role of miR-149 after ischemia remains unclear. We predicted miR-149 targets with the help of Target Scan

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