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Research article

# Up-regulation of Sirtuin-1/autophagy signaling in human cerebral ischemia: possible role in caspase-3 mediated apoptosis

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ABSTRACT

*Aim:* Autophagy is a catabolic process, which plays a pivotal role in neuronal homeostases. Sirtuin-1 (Sirt1, Silent information regulator family protein 1) is a protein deacetylase that is activated by nicotinamide adenine dinucleotide ($NAD^+$), is also influenced by starvation and stress response similar to autophagy. Sirt1 is necessary for the induction of autophagy and is critical for neuronal survival in neurodegeneration. The present study investigates the role of Sirt1/autophagy signaling and its possible involvement in neuronal cell death in the brains of cerebral ischemia (CI) patients.

*Patients and methods:* Autopsied brain tissues from three healthy subjects and ten CI patients were obtained from National Institute of Mental Health and Neurosciences (NIMHANS); Bangalore, India. Western blotting and immunostaining were performed to assess the expression changes in Sirt1, autophagy mediators including Beclin-1, autophagy-related (Atg) proteins-3, 5, 7, 12-5, microtubule-associated protein-1A light chain3 (Lc3-I/II), and caspase-3 in stroke patients.

*Results:* Our study showed that, in stroke patients, expression of Sirt1, Beclin-1, Atg-3, 5, 7, 12-5, and Lc3-II/I were upregulated. Further, our immunohistochemistry results show increased immunoreactivity of Sirt1, Beclin-1, Atg-7, Lc3-I/II, and cleaved caspase-3 in stroke brains.

*Conclusion:* The present data suggesting a role for Sirt1/autophagy signaling in regulating neuronal cell survival in CI.

## 1. Introduction

Ischemic stroke (IS)/cerebral ischemia (CI) remains the primary cause of human mortality and long-term disability [1]. It becomes a global economic and healthcare burden. Apoptosis and necrosis are extensively studied cell death mechanisms attributed to neuronal cell loss in CI. Recent studies demonstrated the activation of autophagy following CI [2]. Basal autophagy is neuroprotective, while excessive/defective autophagy may lead to neuronal death in experimental stroke models [3]. However, the contribution of autophagy to neuronal survival/death in human CI remains elusive.

Autophagy is a conserved cytoprotective process that engulfs long-lived cytoplasmic proteins and defective organelles and transports them to lysosomes for degradation [2]. A series of autophagy-related (Atg) proteins are involved in autophagosome formation and development. Beclin-1 activation initiates the autophagy signaling in response to metabolic and cellular stress. After nucleation, Atg12-5 complex and Lc3-I/II ubiquitin-like conjugation systems are required to expand the phagophore. Atg-7 initiates the activation of Atg-12-5 complex, which then binds with Atg-16L1 to form the Atg-12-5-16L1 complex. The second conjugation system Lc3-I/II, which exists in two different forms, one is cytosolic Lc3-I, the other one is phosphatidylethanolamine (PE) conjugated Lc3-I. This conjugation depends on Atg-4, Atg-7, Atg-3, and Atg-12-5-16LI complex, which leads to the formation of Lc3-II, degrades after lysosome fusion or is separated after binding to the autophagosome membrane. As a result, Lc3-II is an important indicator of the cytosolic autophagosome pool [4]. Recent studies reported that autophagy induction contributes to the neuroprotection in Sirt1-dependent manner in Parkinson's disease and prion protein-mediated neurotoxicity, indicating a correlation between Sirt1 activation and autophagy induction [5, 6].

Mammalian Sirt2 ortholog Sirt1 is a class-III deacetylase that hydrolyzes histones and non-histone proteins to remove the acetyl group from lysine. Sirt1 consumes one $NAD^+$ moiety per acetyl group removed [7]. In lower eukaryotes, Sirt1 mediates caloric restriction-induced longevity

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[8]. It regulates a myriad of transcription factors that mediates inflammation, metabolism, and apoptosis [9, 10, 11]. Evidence from pre-clinical studies indicate a neuroprotective role of Sirt1 against several neurodegenerative disorders [12]. In experimental stroke models, Sirt1 activation has been shown to reduce brain infarction and neuronal injury [13, 14, 15, 16]. Sirt1 can deacetylate various autophagy mediators, thereby controlling autophagy induction [17]. This study investigated Sirt1-induced autophagy signaling in neuronal cell survival after CI in human brain tissues.

## 2. Results

### 2.1. Cerebral ischemia up-regulates Sirt1/autophagy signaling in human brain

Sirt1 protein expression, evaluated using immunoblotting, was increased in the brains of stroke patients compared to healthy controls, but this was not statistically significant (Figure 1A). To understand the involvement of autophagy in CI pathogenesis, the changes in the expression of autophagy proteins including Beclin-1, Atg-3, 5, 7, 12-5, and Lc3-II/I were examined using western blotting. In stroke patients, the expression of autophagy mediators was increased compared to healthy subjects, but the increase was not statistically significant (Figure 1B–G).

### 2.2. Cerebral ischemia increases Sirt1, Beclin-1, Atg-7, and Lc3-I/II immunoreactivity in human brain

To determine the subcellular distribution of Sirt1, autophagy mediators (Beclin-1, Atg-7, and Lc3-I/II), we immunostained paraffin-embedded human brain sections and found increased Sirt1, Beclin-1, Atg-7, and Lc3-I/II, immunostaining in the brains of CI patients compared with normal controls (Figure 2A–D). Further, triple immunofluorescence labeling of Sirt1, Lc3-I/II, and DAPI showed an increase in Lc3-I/II immunostaining that co-located with Sirt1 in the brains of CI patients when compared with normal controls (Figure 2E).

### 2.3. Cerebral ischemia increases pro-apoptotic caspase-3 immunostaining in human brain

Routine H&E staining was performed to show the neurodegeneration in stroke brains (Figure 3A). To confirm the apoptosis, brain sections were immunostained against cleaved caspase-3. We found an increase in cleaved caspase-3 positive cells in the brains of stroke patients compared to healthy controls (Figure 3B). Our results indicate that Sirt1-mediated autophagy singling and pro-apoptotic caspase-3 were found to be upregulated in human brains following stroke.

## 3. Discussion

To the best of our knowledge, this is the first study on the expression of Sirt1/autophagy signaling in the brains of ischemic stroke patients. Our study findings provide new evidence that Sirt1/autophagy signaling is increased in the brains of stroke patients after CI. Further, we observed an increase in immunoreactivity of cleaved caspase-3. The coincident timing of the post-ischemic up-regulation of Sirt1/autophagy signaling and pro-apoptotic caspase-3 suggests that Sirt1/autophagy signaling may affect neuronal survival after CI in human patients.

The autophagic removal of protein aggregates and dysfunctional mitochondria is essential for post-mitotic neuronal cell survival. Autophagy is constitutively active, and the autophagosome clearance system is highly efficient in neurons [18]. Autophagy assists in supporting

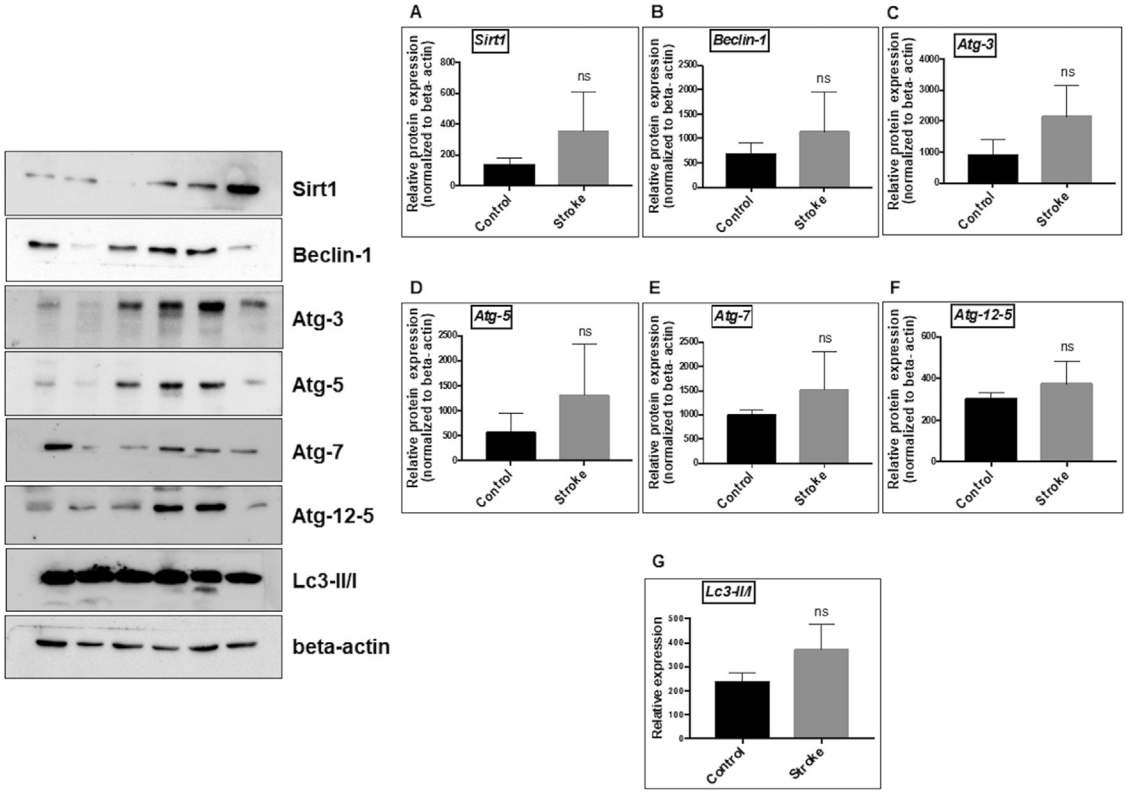


**Figure 1.** Cerebral ischemia up-regulated the expression of Sirt1/autophagy signaling in autopsied brains of stroke patients. Immunoblotting was performed on an equal amount of total protein sample (75 μg ) from healthy controls (n = 3) and stroke patients (n = 6). Respective total proteins and beta-actin were served as a loading control (Lc3-II/I), while Sirt1, Beclin-1, Atg-3, Atg-5, Atg-7, and Atg-12-5 were normalized to beta-actin (A–G). Graphs show changes in the expression of Sirt1/autophagy signaling. The densitometry values are expressed as mean ± SD (Raw immunoblot images were included in this article as supplementary Data).

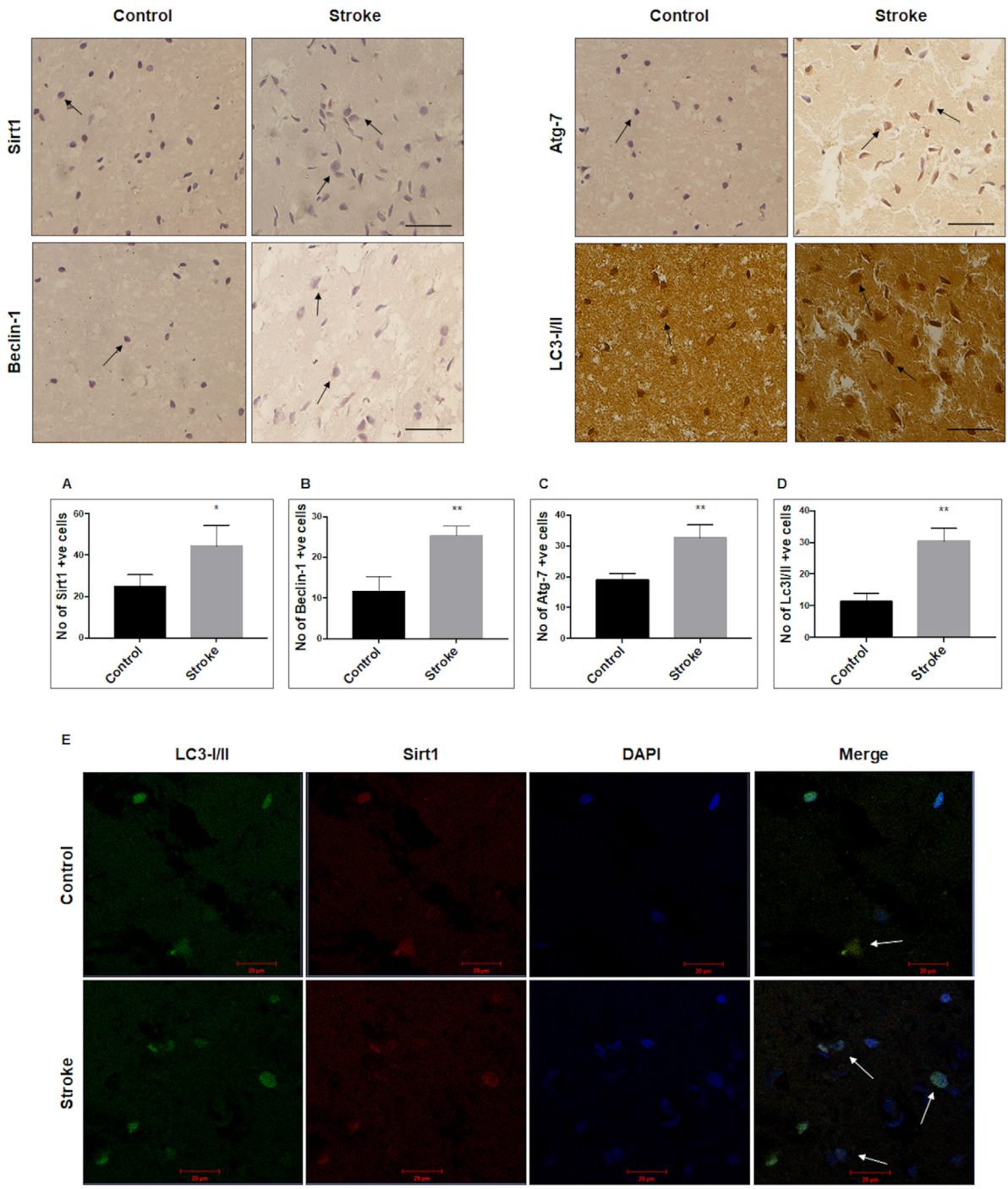


**Figure 2.** Subcellular localization of Sirt1, Beclin-1, Atg-7, and Lc3-I/II in autopsied brains of stroke patients. Brain tissues from healthy subjects (n = 3) and stroke patients (n = 4) were paraffin-embedded and sectioned into 5–10 μm thick slices. (A–D) Graphical representation of changes in Immunoreactivity of Sirt1, Beclin-1, Atg-7, and Lc3-I/II from stroke patients and healthy subjects. The black arrow represents Sirt1, Beclin-1, Atg-7, and Lc3-I/II immunoreactivity for the respective group. Immunoreactivity was depicted in brown (DAB). The neuronal nucleus is counterstained with hematoxylin (magnification 400X; Scale Bar – 20 μm). (E) Triple immunofluorescence labeling for Lc3-I/II (green), Sirt1 (red), and DAPI (blue) in control (n = 3) and stroke human brains (n = 4). The representative image shows increased number of Lc3-I/II positive cells co-localized with Sirt1 in stroke brain compared to control (White arrows; magnification 630X; Scale Bar – 20 μm).

neuronal plasticity and blood-brain barrier integrity [19, 20]. Recent studies reported that mice lacking Atg-5/7 exhibit protein aggregation and motor dysfunction, suggesting that basal autophagy is essential for neuronal homeostasis [21, 22]. However, defective/excessive autophagy is one of the contributing factors for developing various human neurodegenerative disorders. The ultra-microscopic investigation of brain tissue from Alzheimer's and Parkinson's patients showed the accumulation of autophagic vesicles, protein aggregates and damaged mitochondria, indicating failure in autophagic flux [23, 24]. Further, impairment in lysosomal clearance underlies autophagic dysfunction in Niemann-Pick Type C disease [25]. Moreover, in experimental stroke models, defective/excessive autophagy is well documented [2, 3, 26].

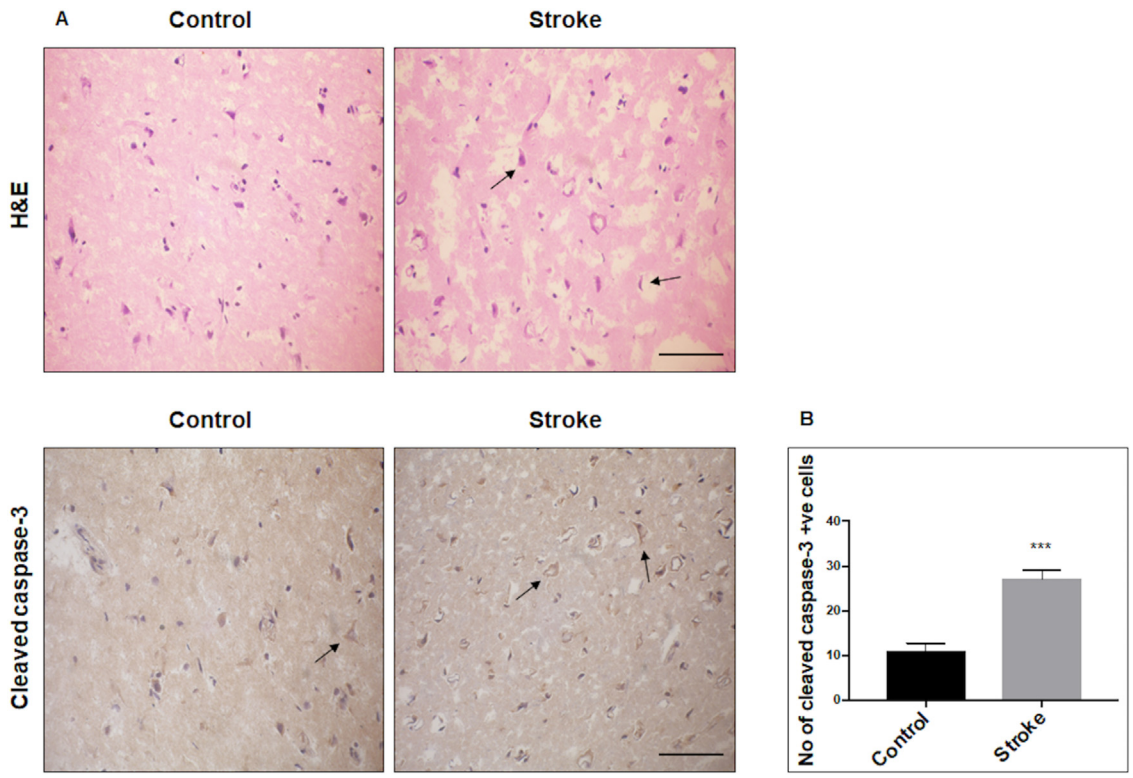


**Figure 3.** H&E staining and subcellular localisation of cleaved caspase-3 in autopsied brains of stroke patients. Brain tissues from healthy subjects (n = 3) and stroke patients (n = 4) were paraffin-embedded and sectioned into 5–10 μm thick slices. (A) H&E staining shows the changes in neuronal morphology (black arrow) in the brains of stroke patients (magnification 400×; Scale Bar – 50 μm). The black arrow represents pyknotic cells in the brains of stroke patients. (B) Graph represents changes in immunoreactivity of cleaved caspase-3 from stroke patients and healthy subjects. Black arrow represents changes in cleaved caspase-3 immunoreactivity for the respective group. The cell count was performed manually using Image J software (NIH; US). Immunoreactivity was depicted in brown (DAB). The neuronal nucleus is counterstained with hematoxylin (magnification 400×; Scale Bar – 20 μm).

Our results show increased autophagy in stroke patients. The failure in cargo recognition and clearance mechanism might be the reason for the increased autophagy in human stroke patients [27, 28].

Autophagy regulation is organ and tissue-specific. The heart, skeletal muscle, and pancreas express high levels of Lc3-II. The brain shows elevated levels of Lc3-I and limited Lc3-II expression, implying that the brain's primary ability can respond rapidly with a change in autophagosomal pool size without requiring *de novo* Lc3 synthesis [29]. Our immunoblot results show higher levels of Lc3-I and limited Lc3-II expression in control subjects and increased Lc3-II/I ratio in stroke patients.

Sirt1 is more abundant in the adult brain and neurons [30, 31]. It involves in neuronal plasticity and memory [30, 32]. Sirt1 is shown to protect against CI by regulating p53-mediated neuronal apoptosis [13]. It also mediates hyperbaric oxygen preconditioning induced tolerance to CI through inhibition of cleaved caspase-3 [14]. Sirt1 can directly deacetylate various autophagy mediators, including Atg-5, Atg-7, and Lc-3 [33]. The induction of autophagy confers neuroprotection against CI and prion protein-mediated neurotoxicity in Sirt1 dependent manner [6, 34]. Further, oxymatrine treatment attenuates cognitive deficit through Sirt1 mediated autophagy in CI rats [35]. The neuroprotective role of Sirt1 against ischemia is controversial. Study with Sirt1 overexpression mice shows a reference memory deficit, but not neuroprotection against brain ischemia [36]. Our recent study found high Sirt1 expression in the brain of CI patients [37]. The present study shows increased Sirt1 expression in the brains of stroke patients. The discrepancy in our results with pre-clinical stroke models might be due to different factors that regulate Sirt1 functional activity, such as subcellular localization, $NAD^+$ pool's availability [12], and the stress stimuli intensity [38].

Activation of apoptosis is the major contributor to neuronal damage in CI [39, 40]. Autophagy can prevent or activate apoptosis following IS [41, 42]. Bcl-2/Beclin-1 interaction serves as a crossroad to lead neuronal cells to apoptosis or autophagy [43]. Autophagy mediators Atg-5, and Atg-7 can promote apoptosis induction independent of their role in autophagy. Further, caspase-3 activation was inhibited in neuron specific Atg-7 knockout mice [44]. Caspase-3 can affect various autophagy mediators such as Beclin-1, Atg-4d, and Atg-5 by direct interaction [45]. Interestingly, we found increased immunoreactivity of cleaved caspase-3, indicating that autophagy and apoptosis can cross talk and regulate many common death mediators. The functional relationship between Sirt1, autophagy, and apoptosis is complex and depends on the cellular environment, intensity of stress signal, and different effector pathways that

**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 | 65/F | 12 h 30 min | Necrotic tissue right infarct |
| 6 | 43/M | 4 h | Right MCA territory 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.

share Sirt1/autophagy and autophagy/apoptosis pathway like forkhead box class O (FoxO), p53pathway, p62, and Atg-7. The major limitation of this study is the small sample size. However, our results offer a promising basis from which can be conducted to further elucidate the role of Sirt1/autophagy signaling and its involvement in neuronal cell death.

## 4. Conclusion

The present study concludes that CI upregulated Sirt1/autophagy signaling and caspase-3 in human stroke patients, suggesting that activation of Sirt1/autophagy signaling may promote caspase-3 mediated cell death or may act as a rescue mechanism to prevent neuronal cells from succumbing apoptotic death following CI. However, Sirt1/autophagy signaling may represent a suitable therapeutic target for the treatment of CI.

## 5. Materials and methods

### 5.1. Reagents and chemicals

All the antibodies used in this study were purchased from Cell Signaling Technology (CST, US). Sirt1 (CST#8649), Autophagy Antibody Sampler Kit (CST#4445), beta-actin (CST#8457), Anti-rabbit Ig-G-Alexa fluor-488 (CST#4412), Anti-mouse Ig-G Alexaflour-555 (CST# 4409), Prolong Gold Antifade Reagent with DAPI (CST#8961).

### 5.2. Post-mortem human brain tissues

An autopsy cohort of control (n = 3) and ischemic stroke (n = 10) human brain tissues were acquired from "Human Brain Bank, Department of Neuropathology, National Institute of Mental Health and Neurosciences (NIMHANS); Bangalore, India." The Institutional Ethical Committee (IEC), University of Hyderabad, India (UH/IEC/2019/92) approved all experimental procedures on human tissues in agreement with the 1964 Helsinki declaration and its subsequent amendments or comparable ethical standards. Informed consent was obtained from the family members of the patient. Patient information, including infarct region, age and sex are given in Table 1.

### 5.3. Immunoblotting

Control and stroke patient brain tissues were homogenized in lysis buffer (RIPA buffer: 50 mM Tris-HCl (pH-7.4), 2 mM EDTA, 150 mM NaCl, 10% Glycerol, 0.5% Sodium deoxycholate, & 1% NP40). Tissue homogenates were centrifuged at 10,000 rpm for 20 min to separate supernatant. The Lowry method was used to determine the protein concentration in the supernatant. Western blots were used to examine an equal amount of total protein sample (75μg). The total protein lysates were separated on 10–12% SDS-PAGE gels before being transferred to nitrocellulose membrane (NC). NC membranes were incubated for 90 min at room temperature (RT) with 5% skimmed milk powder in Tris-buffered saline with Tween-20 (TBS-T). Next, membranes were incubated overnight at 4 °C with primary antibody (1:1000 dilution) against Sirt1, Beclin-1, Atg-3, Atg-5, Atg-7, Atg-12-5, Lc3-I/II, and beta-actin. Membranes were washed with TBS-T after primary antibody incubation and incubated with HRP labeled secondary antibody (rabbit specific) for 90 min at RT (1:3000 dilution). ECL detection system was used to capture chemiluminescence (Bio-Rad, USA).

### 5.4. Immunohistochemistry

Paraffin-embedded brain tissues from control and stroke patients were sectioned into 5–10 μm thick slices using a microtome (Leica, Germany). Brain sections were immunostained against Sirt1, Beclin-1, Atg7, Lc3-I/II, and cleaved caspase-3 using immunohistochemistry detection kit (Bio SB, USA). Sections were deparaffinized in three 5 min xylene changes before being hydrated in an 8 min alcohol gradient. Brain sections were covered with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidase. After three washes in PBS, brain sections were covered in Tris-EDTA buffer for heat-induced antigen retrieval (Microwave boiling three times, each 5 min with 1 min interval). After PBS washes, brain sections were covered for 30 min with 0.25% BSA followed by primary antibody (1:100 dilution) incubation for 2 h 30 min at RT. After three washes in PBS, brain sections were incubated in respective HRP-labelled secondary antibody (provided with the kit) for 45 min at RT. Staining was made visible with 3′-3′ diaminobenzidine (DAB) buffer (supplied in kit) for 5 min, followed by counterstaining by hematoxylin. Brain sections were mounted after dehydration, and images were taken using a light microscope (Olympus, Japan) at 400 X magnification.

### 5.5. Hematoxylin & eosin (H&E) staining

Control and stroke brain sections were dewaxed three times in xylene before being hydrated in a decreased alcohol gradient. Brain sections were washed three times in PBS and stained with hematoxylin, followed by staining with eosin. Next, brain sections were dehydrated in an increased alcohol gradient, cleared in xylene, and mounted. Brain sections were examined under the light microscope (Olympus, Japan) for changes in neuronal cell morphology at 400× magnification.

### 5.6. Immunofluorescence

Triple immunostaining was performed to probe for Sirt1, Lc3-I/II and DAPI. Brain sections from control and stroke patients were dewaxed and rehydrated through 100-80% graded ethanol in distilled water. Brain sections were transferred to citrate buffer for heat-induced antigen retrieval (boiled three times, each 5 min in a microwave). After three PBS washes, sections were blocked for 60 min at 37 °C with 5% goat serum. Brain sections were then separately incubated with primary rabbit antibodies against Lc-3I/II, mouse antibody against Sirt1 (1:100 dilution) overnight at 4 °C. After PBS washes, brain sections were incubated with Alexa Fluor-485 conjugated goat anti-rabbit and mouse IgG (1:3000 dilutions) for 60 min at 37 °C. After washing the slides in PBS, they were mounted with antifade DAPI and imaged using a laser scanning confocal microscope (Carl-Zeiss, Germany).

### 5.7. Statistical analysis

The data were presented as the mean ± SD and analyzed for statistical significance using an unpaired t-test. The experimental data shown are representative of three independent experiments. Bars represent variation within the experimental samples. Graphs were created using GraphPad prism 7.0 software (GraphPad Inc. US). $p > 0.05$ indicates no significance (ns).

## Declarations

### Author contribution statement

Sireesh Kumar Teertam, Ph. D: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Phanithi Prakash Babu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### Data availability statement

Data included in article/supp. material/referenced in article.

### Declaration of interest's statement

The authors declare no conflict of interest.

### Additional information

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