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Evidence of Post-translational Modifications of Proteins in Clinical Samples of Stroke after Decompressive Craniectomy

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ABSTRACT: Stroke is the leading cause of mortality and disability the world over. In India, stroke stands as the fourth leading cause of death. Currently, no treatment strategies exist for stroke except for thrombolytics. Stroke pathology is complex that activates multiple mechanisms of cell death pathways, thus requiring extensive investigation. The present study focused on the activation of post-translational modifications (PTMs) of proteins that decides the fate of neuronal death, such as protein ubiquitination, SUMOylation, and phosphorylation, in clinical samples of stroke collected from patients who underwent decompressive craniotomy or craniectomy (DC). Tissues of brain cortex were collected from patients after emergency DC with large Middle Cerebral Artery (MCA) infarcts. We used brain cortex tissue samples collected after MCA occlusion in rats to analyze protein ubiquitination. We performed histological analysis using hematoxylin and eosin and immunofluorescence staining. We report the following critical findings in clinical samples of stroke: 1. Altered morphological features of necrosis, 2. A pronounced expression of SUMO-2/3 protein confined to the nucleus, indicating a possible role in modulating gene expression, 3. Accumulation of abnormal or ubiquitinated proteins, 4. Phosphorylation of eukaryotic initiation factor 2 alpha (peIF2α) is one of the hallmarks of endoplasmic reticulum (ER) stress or unfolded protein response. The present study using clinical samples collectively indicates that PTMs are associated with stroke-induced brain damage and provide insights into understanding stroke pathophysiology and help develop newer therapeutic strategies aiming at stroke/cerebral ischemia to curtail brain damage.

Keywords: Stroke, Ubiquitin, SUMO, peIF2α, Neuroprotection

INTRODUCTION

Stroke is the leading cause of mortality (stands fourth) and disability (stands fifth) in India, while the world-over statistics indicate it stands second and third, respectively (Jones *et al.,* 2022; Feigin *et al.,* 2022). The thrombolytic agent (alteplase or recombinant tissue plasminogen activator) remains the only therapy approved for acute stroke with limitations such as a narrow therapeutic window and bleeding complications (Mosconi *et al.,* 2022). The pathophysiology of stroke is complex and leads to the activation of multiple cell death pathways in the post-stroke brain (Nakka *et al.,* 2008; Nakka *et al.,* 2016). Thus understanding multiple mechanisms of cell death and correlating the same in clinical samples might provide better insights into newer therapies for stroke.

Ubiquitin (Ub) and Ub-like post-translational modifications (PTMs), such as small ubiquitin-related modifier (SUMO), are critical players underlying stroke damage (Nakka *et al.,* 2020). Increased protein ubiquitination was reported during reperfusion in the post-ischemic cortex (Hochrainer *et al.,* 2012). The cortex is considered an ischemic penumbra (where the brain cells are on the verge of dying or undergoing delayed neuronal death). Thus targeting the cortex might help to understand stroke pathophysiology and insights into drug development (Liu *et al.,* 2010). SUMOylation (addition of SUMO to target proteins), which is similar to ubiquitination but with a distinct functional significance such as DNA repair, neuroprotection, degradation of proteins, etc. Conjugation of SUMO-2/3 subtypes is prominently expressed in the ischemic cortex with a nuclear translocation (Yang *et al.,* 2008). Further, reports indicate a possible crosstalk between ubiquitination and SUMOylation during cerebral ischemia and reperfusion; however, the outcome of such interactions is not clearly understood (Nakka *et al.,* 2020). Stroke-induced endoplasmic reticulum (ER) stress or unfolded protein response (UPR) leads to the

phosphorylation of eukaryotic initiation factor 2 alpha (peIF2α), one of the forms of PTMs and pharmacological regulation of eIF2α phosphorylation showed neuroprotection after MCAO (Nakka *et al.,* 2010; Nakka *et al.,* 2022). Phosphorylation of eIF2α is a downstream event of one of the activated UPR pathways, Protein Kinase RNA-Like ER Kinase (PERK) pathway (Nakka *et al.,* 2022; Raghubir *et al.,* 2011). Both ubiquitination and SUMOylation are reported to regulate ER stress response (Qu *et al.,* 2021; Lim *et al.,* 2014). Thus the pathophysiology of stroke seems complex, involving distinct yet overlapping pathways. The present study might provide some critical insights into PTMs from the bench to the clinic in developing therapies for stroke.

METHODS

Compliance with Ethical Standards: We conducted experiments using clinical samples of stroke after obtaining approval and according to the guidelines of the Institutional Ethics Committee (approval reference number: GMC/IEC/100/2018) from Guntur medical college & Government general hospital, Andhra Pradesh-522004, India. We obtained informed consent from patients.

Collection of brain tissue samples: Brain tissue samples were obtained while performing emergency decompressive craniotomy or craniectomy (DC) in patients (males; aged between 49-55 years) with large Middle Cerebral Artery (MCA) infarcts (n=3). Of note, damaged areas are already ischemic. We obtained traumatic brain injury samples (used as a control for MCA infarct tissue) from the right frontal lobe with a contused brain (around 0.3cms x 0.3cms) to use as a control (one female and two males; aged between 24-50 years), and removal of such amount of tissue does not cause any significant damage to the brain and patients in these cases. DC is a well-known surgical procedure to manage intracranial pressure for acute stroke (Beez *et al.,* 2019).

Histological analysis: We made paraformaldehyde-fixed and paraffin-embedded brain tissue wax blocks soon after collection and preserved them till further processing. We collected paraffin-embedded microtome sections of 8 to10µ thickness on either Silane-Prep (Sigma-Aldrich) or albumin-coated slides for hematoxylin and eosin (H&E) staining and Immunofluorescence.

Haematoxylin and eosin (H&E) staining*:* Brain tissue samples were externally fixed in 4% paraformaldehyde prepared in phosphate-buffered saline. We performed H&E staining as described earlier with minor modifications (Li *et al.,* 1998). In brief, sections were stained with haematoxylin (5 min) and eosin (1 min), followed by dehydration and mounting with DPX. Stained brain sections were examined microscopically for morphological abnormalities such as necrosis and apoptosis.

Immunofluorescence. We performed immunofluorescence staining as described earlier with slight modifications (Zaqout *et al.,* 2020; Nakka *et al.,* 2010). In brief, procedures such as dewaxing, heat antigen retrieval using 10mM Sodium citrate buffer, cell permeabilization using Triton X-100, and blocking in 1% BSA. Brain sections were incubated with primary antibodies overnight at 4° C, and antibody details are as follows. Anti-Ubiquitin (Catalog # 13-1600; Invitrogen/Thermo-Scientific), anti-SUMO-2/3, and Phospho-eIF2α (Ser51) antibody (Cat#4971T and Catalog#9721, respectively, from Cell Signalling Technology (CST), USA. We used corresponding secondary antibodies (Catalog # A32731; Alexa Fluor Plus 488; Invitrogen/Thermo-Scientific) and counterstained with ProLongTM Gold Antifade Mountant with DAPI (Catalog # P36941; Thermo-Scientific). We used a fluorescence microscope (Olympus 1X81) to capture images and analysis.

Middle Cerebral Artery Occlusion (MCAO) in rats: We conducted animal experiments according to the institutional animal ethics committee guidelines (ANUCPS/IAEC/AH/P/10/2018). Transient focal cerebral ischemia was induced by MCAO in male Sprague–Dawley rats (weighing 250-270 grams) using 3–0 nylon monofilament (from Ethicon, Johnson & Johnson Ltd.) as previously described (Nakka *et al.,* 2022; Nakka *et al.,* 2010; Longa *et al.,* 1989). In brief, we occluded the left MCA for 60min, followed by reperfusion. MCAO is a well-known animal model that reiterates human thrombo-embolic strokes.

Western blot analysis: We performed western blot analysis as described previously (Nakka *et al.,* 2010; Nakka *et al.,* 2022). In brief, we loaded an equal amount of protein (100µg) in each well to detect Ubiquitin conjugates in the post-ischemic cortex tissue using an antibody that detects both conjugated and free Ubiquitin (Catalog # 13-1600; Invitrogen/Thermo-Scientific). As a protein loading control, we used β-actin (Antibody catalog # 8457 from CST). Corresponding secondary (HRP conjugated) were used at 1:5000 dilutions (from CST) to detect protein bands/aggregates using either West Pico PLUS Chemiluminescent kit (Thermo-Scientific).

RESULTS

The present study provides evidence that PTMs such as ubiquitination, SUMOylation, and selective phosphorylation of eIF2α occur in the post-ischemic cortex samples of the human brain collected after emergency DC. PTMs play a significant role in determining the fate of brain cell death in animal models of experimental stroke. This study attempted to correlate and validate the pre-clinical observations in clinical samples of stroke. PTMs such as ubiquitination, SUMOylation, and phosphorylation of eIF2α play

critical roles in the post-ischemic brain (Nakka *et al.,* 2020; Nakka *et al.,* 2022).

Neuronal morphology alters in the post-ischemic cortex. We have observed significant changes in the cellular morphology in the human ischemic cortex samples. The changes appeared necrotic with characteristic features such as hypoxic and pyknotic nuclei, indicating the severity of neuronal damage (Fig. 1). Some of the neurons within the ischemic cortex exhibited apoptotic-like morphology, such as the condensed nucleus and apoptotic bodies (Fig. 1 lower panel). Most neurons undergo necrosis in the ischemic core region, where the damage is severe or irreversible. Apoptosis is a programmed cell death seen in delayed neuronal death (Kaufmann *et al.,* 1999; Radak *et al.,* 2017). PTMs such as ubiquitination, SUMOylation, and phosphorylation of eIF2α linked to apoptosis regulation (Seyrek *et al.,* 2020; Schubert *et al.,* 2000).

Formation of Ubiquitin protein aggregates/ Ubiquitination occurs in the post-ischemic stroke cortex. Ubiquitination plays a critical role in the pathophysiology of stroke by carrying out selective and non-selective protein degradation depending on the severity of the stroke. Limited or selective ubiquitination leads to neuroprotection; however, severe or prolonged stress causes neuronal death due to an overload of proteasome or aggregation of abnormal proteins. We have observed an intense staining pattern of protein ubiquitination in the human ischemic cortex indicating active PTMs are part of ischemic brain damage (Fig. 2 upper panel). We assessed the time-dependent activation of ubiquitination during reperfusion (6h, 12h, and 24h) after MCAO (1h) in rats. Cerebral ischemia/reperfusion caused increased protein ubiquitination in the ischemic cortex compared to the sham group (Fig. 3). Thus ubiquitination in the human ischemic cortex correlates preclinical outcome of the post-ischemic brain.

SUMO-2/3 expresses in the post-ischemic stroke cortex. Similar to the ubiquitination SUMOylation also play a critical role in the stroke pathophysiology. We used specific antibodies to detect SUMO-2/3 expression in the human ischemic cortex mostly confined to the nucleus consistent with DAPI staining specific to the nucleus (Fig. 2 lower panel). Thus it indicate that ubiquitin–like PTMs such SUMOylation might regulate pathophysiology of stroke in humans.

The eIF2α undergoes phosphorylation in the post-ischemic cortex. Stroke-induced ER stress/UPR plays a critical role in rodent MCAO models (Nakka *et al.,* 2010; Nakka *et al.,* 2022). The UPR-activated PERK pathway phosphorylates its downstream effect or molecule eIF2α, which temporarily inhibits protein synthesis and copes with the ER stress and protein aggregation and misfolding. We used specific antibodies that detect phosphorylated eIF2α at Ser51 amino acid residue. We observed the expression or staining of eIF2α phosphorylation prominent in the human ischemic cortex (Fig. 4). Thus our observation indicates the activation of ER stress or UPR branch PERK- eIF2α pathway in the human ischemic cortex. Of note, limited phosphorylation is beneficial, while aberrant or sustained phosphorylation of eIF2α could be detrimental to the survival of neurons in the ischemic cortex.

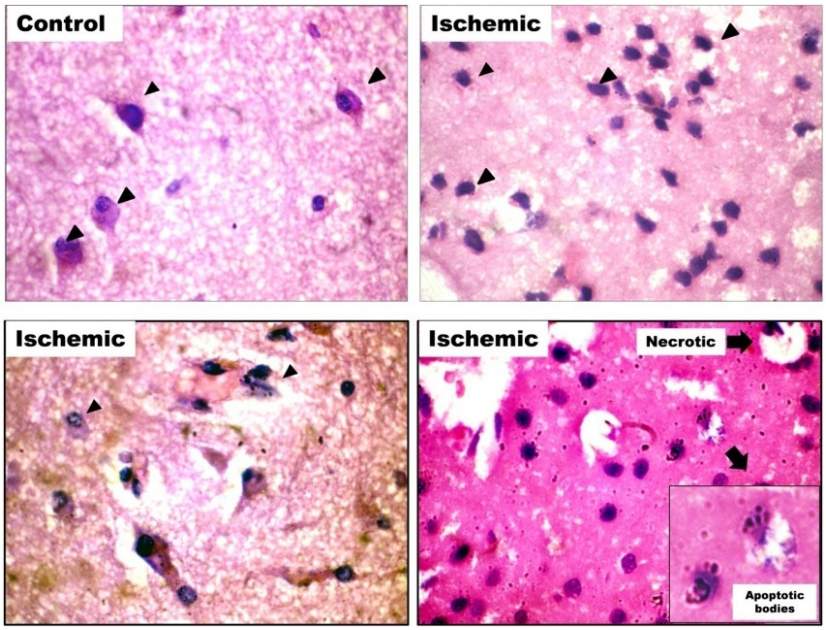

Fig. 1. H&E staining shows morphological alterations in the human ischemic cortex tissue sections (representing three individual tissue samples). We observed pronounced distortion of neurons exhibiting characteristic features of necrosis (upper right and lower left panels) and apoptotic-like features such as condensation of the nucleus and formation of apoptotic bodies (highlighted in the inset of the lower right panel). We also observed features of mixed necrosis and apoptosis (lower right panel). The original magnification of the images is x400.

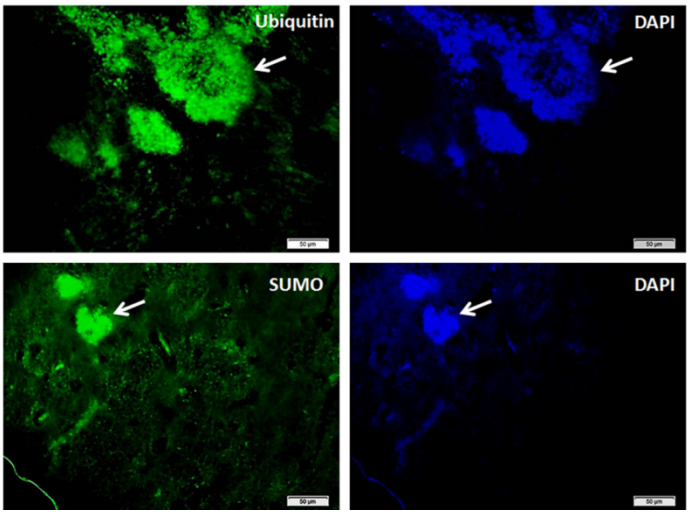

Fig. 2. Shows immunofluorescence staining of ubiquitin (FITC- green) in the human ischemic cortex (upper panel), and SUMO-2/3 staining (FITC- green) in the human ischemic cortex (lower panel). We counterstained sections with DAPI (blue). We represented the result from three individual tissue samples of the ischemic cortex.

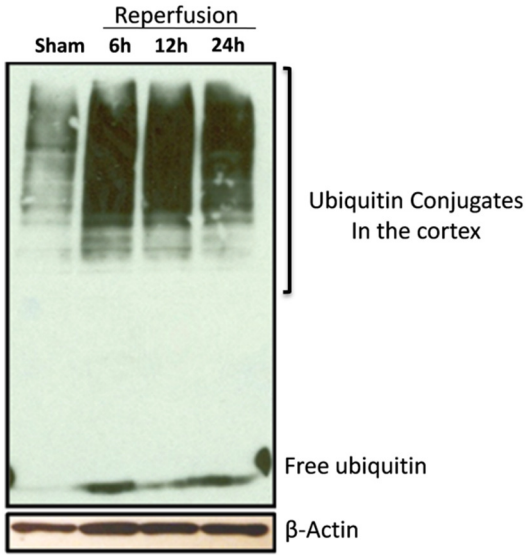

Fig. 3. Shows accumulation of ubiquitinated protein aggregates in the brain cortex following different time points of reperfusion after MCAO in rats compared to sham control (n=3 per group). Prolonged protein aggregates lead to the activation of unfolded protein response/ER stress, neuronal cell death, and proteasome dysfunction.

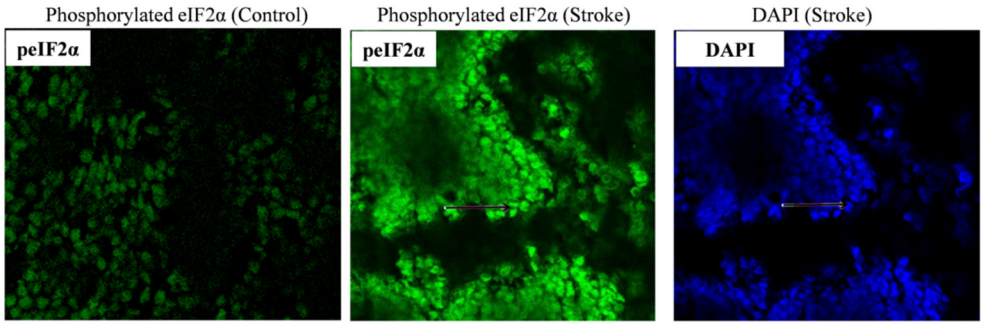

Fig. 4. Shows immunofluorescence staining of eIF2α phosphorylation (FITC- green) in the human ischemic cortex. We counterstained sections with DAPI (blue) to visualize the nucleus. The ischemic cortex shows intense immunofluorescence staining compared to the control (pale staining). The original magnification of the images is x400. We represented the result from three individual tissue samples.

DISCUSSION

During protein synthesis, ~30% of proteins undergo quick degradation due to abnormal protein folding and aggregation. Stroke and other neurodegenerative disorders lead to such protein abnormality and hamper the mechanism of protein quality control (Schubert *et al.,* 2000; Mabb *et al.,* 2010). Ubiquitin mediated selective protein degradation seems neuroprotective after brief ischemia; however, severe or prolonged ischemia leads to dysfunction of proteasome and formation of ubiquitinated proteins aggregation (Meller *et al.,* 2009).

In the present study, MCAO in rats resulted in massive ubiquitination of proteins in the ischemic cortex during the reperfusion compared to the sham group. Human ischemic cortex samples collected after DC also showed pronounced expression of ubiquitin aggregates, a positive correlation with pre-clinical data. Protein aggregation has consequences such as activation of prolonged ER stress or UPR leading to neuronal cell death (Nakka *et al.,* 2010). Phosphorylation of eIF2α indicates the activation of ER stress that attempts to resolve abnormal proteins in the stressed ER lumen. We detected intense staining of eIF2α phosphorylation in the human ischemic cortex, indicating the activation of ER stress or the UPR branch of the PERK pathway. Our recent reports on animal models of stroke provide substantial evidence regarding the role of eIF2α phosphorylation. We reported that selective pharmacological inhibition of eIF2α dephosphorylation against acute ischemic insult is neuroprotective (Nakka *et al.,* 2010). However, sustained levels of eIF2α phosphorylation promote apoptosis, and administering potent anti-oxidants such as apocynin curtails multiple cell death activators via inhibiting accumulation of reactive oxygen species, protein aggregation, and ER stress or UPR (Nakka *et al.,* 2022). Morphological analysis of the human ischemic cortex featured many necrotic cells, which indicate irreversible damage to the cells; we also observed apoptotic cell features such as condensed nuclei, cell shrinkage, apoptotic bodies, etc. Thus pharmacological regulation of PTMs, such as protein ubiquitination and eIF2α phosphorylation, might help in developing neuroprotective strategies.

In the present study, ubiquitin-like post-translational modification SUMOylation (SUMO-2/3 conjugates) also showed prominent staining in the human ischemic cortex. SUMOylation exerts functions distinct from ubiquitination (Hay, 2005; Silveirinha *et al.,* 2013). Indeed, Increased SUMOylation in transgenic rodent models of MCAO resulted in smaller ischemic infarcts (Lee *et al.,* 2011). Normal physiological functions of SUMOylation include embryonic development, remodelling of chromatin, gene expression, signal transduction pathways, etc. (Bernstock *et al.,* 2018).

CONCLUSIONS

PTMs critically regulate the fate of neuronal cell death and survival in the post-stroke brain. Stroke leads to protein abnormality and hampers the protein quality control mechanism, which is detrimental to the survival of neurons. Our study in human stroke samples (brain cortex) provides substantial evidence and a possible role for PTMs such as ubiquitination, eIF2α Phosphorylation (a marker of ER stress or UPR), and SUMO-2/3) that play a critical role in regulating post-ischemic neuronal death. This evidence provides better insight into understanding the pathophysiology underlying stroke damage.

FUTURE SCOPE

A detailed investigation of PTMs activation and mechanisms at the molecular level in more clinical samples in post-stroke brain provide better insight into novel therapeutics aiming for stroke.

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Conflict of Interest. None.

REFERENCES

Bernstock, J. D., Yang, W., Ye, D. G., Shen, Y., Pluchino, S., Lee, Y. J., Hallenbeck, J. M. and Paschen, W. (2018). SUMOylation in brain ischemia: Patterns, targets, and translational implications. *Journal of Cerebral Blood Flow and Metabolism*, *38*(1), 5-16.

Beez, T., Munoz-Bendix, C., Steiger, H. J. and Beseoglu, K. (2019). Decompressive craniectomy for acute ischemic stroke. *Critical Care*, *23*(1), 209.

Feigin, V. L., Brainin, M., Norrving, B., Martins, S., Sacco, R. L. and Hacke, W. (2022). World Stroke Organization (WSO): Global Stroke Fact Sheet 2022. *International Journal of Stroke*, *17*(1), 18-29.

Hay, R. T. (2005). SUMO: a history of modification. *Molecular Cell*, *18*(1), 1–12.

Hochrainer, K., Jackman, K., Anrather, J. and Iadecola, C. (2012). Reperfusion rather than ischemia drives the formation of ubiquitin aggregates after middle cerebral artery occlusion. *Stroke*, *43*(8), 2229-2235.

Jones, S. P., Baqai, K., Clegg, A., Georgiou, R., Harris, C. and Holland, E. J. (2022). Stroke in India: A systematic review of the incidence, prevalence, and case fatality. *International Journal of Stroke*, *17*(2), 132-140.

Kaufmann, A. M., Firlik, A. D., Fukui, M. B., Wechsler, L. R., Jungries, C. A. and Yonas, H. (1999). Ischemic core and penumbra in human stroke. *Stroke*, *30*(1), 93-99.

Liu, S., Levine, S. R. and Winn, H. R. (2010). Targeting ischemic penumbra: part I - from pathophysiology to therapeutic strategy. *Journal of Experimental Stroke & Translational Medicine*, *3*(1), 47-55.

Lim, Y., Lee, D., Kalichamy, K., Hong, S. E., Michalak, M. and Ahnn, J. (2014). Sumoylation regulates ER stress response by modulating calreticulin gene expression in XBP-1-dependent mode in Caenorhabditis elegans. *The International Journal of Biochemistry & Cell Biology*, *53*, 399-408.

Li, Y., Powers, C., Jiang, N. and Chopp, M. (1998). Intact, injured, necrotic and apoptotic cells after focal cerebral ischemia in the rat. *Journal of Neurological Sciences*, *156*(2), 119-132.

Lee, Y. J., Mou, Y., Maric, D., Klimanis, D., Auh, S. and Hallenbeck, J. M. (2011). Elevated global SUMOylation in Ubc9 transgenic mice protects their brains against focal cerebral ischemic damage. *PloS one*, *6*(10), e25852.

Longa, E. Z., Weinstein, P.R., Carlson, S. and Cummins, R. (1989). Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*, *20*(1), 84-91.

Mabb, A. M. and Ehlers, M. D. (2010). Ubiquitination in postsynaptic function and plasticity. *Annual Review of Cell and Developmental Biology*, *26*, 179-210.

Meller, R. (2009). The role of the ubiquitin proteasome system in ischemia and ischemic tolerance. *Neuroscientist*, *15*(3), 243-60.

Mosconi, M. G. and Paciaroni, M. (2022). Treatments in Ischemic Stroke: Current and Future. *European Neurology*, *85*(5), 349-366.

Nakka, V. P., Gusain, A., Mehta, S. L. and Raghubir, R. (2008.) Molecular mechanisms of apoptosis in cerebral ischemia: multiple neuroprotective opportunities. *Molecular Neurobiology 37*(1), 7-38.

Nakka, V. P., Gusain, A. and Raghubir, R. (2010). Endoplasmic reticulum stress plays critical role in brain damage after cerebral ischemia/reperfusion in rats. *Neurotoxicity Research*, *17*(2), 189-202.

Nakka, V. P., Gogada, R., Simhadri, P. K., Qadeer, M. A. and Phanithi, P. B. (2022). Post-treatment with apocynin at a lower dose regulates the UPR branch of eIF2α and XBP-1 pathways after stroke. *Brain Research Bulletin*, *182,* 1-11.

Nakka, V. P. and Mohammed, A. Q. (2020). A Critical Role for ISGylation, Ubiquitination and, SUMOylation in Brain Damage: Implications for Neuroprotection. *Neurochemical Research*, *45*(9), 1975-1985.

Nakka, V. P., Prakash-Babu, P. and Vemuganti, R. (2016). Crosstalk Between Endoplasmic Reticulum Stress, Oxidative Stress, and Autophagy: Potential Therapeutic Targets for Acute CNS Injuries. *Molecular Neurobiology*, *53*(1), 532-544.

Qu, J., Zou, T. and Lin, Z. (2021). The Roles of the Ubiquitin-Proteasome System in the Endoplasmic Reticulum Stress Pathway. *International Journal of Molecular Sciences*, *22*(4), 1526.

Radak, D., Katsiki, N., Resanovic, I., Jovanovic, A., Sudar-Milovanovic, E., Zafirovic, S., Mousad S. A. and Isenovic, E. R. (2017). Apoptosis and Acute Brain Ischemia in Ischemic Stroke. *Current Vascular Pharmacology*, *15*(2), 115-122.

Raghubir, R., Nakka, V. P. and Mehta, S. L. (2011). Endoplasmic reticulum stress in brain damage. *Methods In Enzymology*, *489,* 259-75.

Silveirinha, V., Stephens, G. J. and Cimarosti, H. (2013). Molecular targets underlying SUMO-mediated neuroprotection in brain ischemia. *Journal of Neurochemistry*, *127*(5), 580–591.

Schubert, U., Antón, L. C., Gibbs, J., Norbury, C. C., Yewdell, J. W. and Bennink, J. R. (2000). Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature*, *404*(6779), 770-774.

Seyrek, K., Ivanisenko, N. V., Richter, M., Hillert, L. K., König, C. and Lavrik, I. N. (2020). Controlling Cell Death through Post-translational Modifications of DED Proteins. *Trends in Cell Biology*, *30*(5), 354-369.

Yang, W., Sheng, H., Homi, H. M., Warner, D. S. and Paschen, W. (2008). Cerebral ischemia/stroke and small ubiquitin-like modifier (SUMO) conjugation–a new target for therapeutic intervention? *Journal of Neurochemistry*, *106*(3), 989–9999.

Zaqout, S., Becker, L. L. and Kaindl, A. M. (2020). Immunofluorescence Staining of Paraffin Sections Step by Step. *Frontiers in Neuroanatomy*, *14*, 582218.

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