Salvianolate lyophilized injection induced autophagy against neuronal apoptosis through the Akt/mTOR pathway in Neuro-2a cells

Dongna Li
Tianjin University of Traditional Chinese Medicine
Mengmeng Ma
Beijing Northen Hospital of Weaponry Industry
Wenqi Zhang
Tianjin University of Traditional Chinese Medicine
Yangyang Xu
Tianjin University of Traditional Chinese Medicine
Shaoxia Wang
Tianjin University of Traditional Chinese Medicine
Lijuan Chai
Tianjin University of Traditional Chinese Medicine
Hong Guo (cacti1983@163.com)
Tianjin University of Traditional Chinese Medicine https://orcid.org/0000-0001-5277-7637
Limin Hu
Tianjin University of Traditional Chinese Medicine

Research

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Abstract

Background: Stroke remains a worldwide health problem. Salvianolate lyophilized injection (SLI) is one of the most widely-used in clinics in China for treating stroke. Our previous research found that SLI protects against stroke by inhibiting oxidative stress and inflammation. Whether SLI has other biological effects on stroke remains unknown.

Methods: Neuro-2a (N2a) cells were treated with SLI (10, 25, 50μg/ml) and exposed to oxygen-glucose deprivation/reoxygenation (OGD/R) as an in vitro model of ischemic stroke. Then, cell apoptosis was assessed using flow cytometry, apoptosis and autophagy related proteins were investigated by western blotting. Autophagy inhibitor 3-methyladenine (3-MA) pretreatment was used to detect the apoptosis effect of SLI on N2a cells.

Results: In this work, we verified that SLI promoted cell proliferation, inhibited cell injury and apoptosis in OGD/R-induced N2a cells. The increase the expression of autophagy markers LC3 and Beclin-1, and decrease autophagy substrate protein p62 demonstrated the induction of autophagy by SLI. Additionally, we found that SLI activation of autophagy significantly preceded inhibition of apoptosis in N2a. Pretreatment with autophagy inhibitor 3-MA could antagonize the protective effect of SLI on inhibiting apoptosis, which may be related to the Akt/mTOR signalling pathway.

Conclusions: The present study indicated that SLI has potential as a novel therapy for ischemic stroke, and its possible mechanism is to induced autophagy and inhibit apoptosis through blocking Akt/mTOR signaling pathway in OGD/R-induced N2a cells, providing new insights into the mechanism of SLI in the treatment of stroke.

Background

Ischemic stroke is a common cerebrovascular disorder disease, not only seriously affects people's physical and mental health, but also causes a huge economic burden on families and society [1, 2]. The previous study revealed that ischemic stroke is characterized by multiple kinds of pathological and physiological changes, including oxidative stress, inflammatory reaction and increased vascular permeability [3–5]. Recent studies have shown that autophagy and apoptosis are simultaneously involved in the pathogenesis of ischemic stroke, where exists interaction between each other [6, 7].

Autophagy, a catabolic process, is a vital phenomenon existing in eukaryotic cells. Autophagy dysfunction is closely associated with certain disease, including nervous system diseases, immune responses, cancer and diabetes [8–11]. Accumulating evidence indicates that autophagy contributes to

cerebral ischemic stroke and is involved in ischemic brain injury [12, 13]. Autophagy is activated in various cell types in the brain such as neurons, glia cells, and brain microvascular cells upon ischemic stroke [14]. During early reperfusion in rats, activating autophagy can provide affirmative neuroprotection against the cerebral ischemia/reperfusion (I/R)-induced injury [15]. Mild hypothermia can reduce the cerebral ischemic injury by activating autophagy and inhibiting pyroptosis [16]. However, autophagy is a 'double-edged sword [17]'. In some special circumstances, excessive autophagy could lead to cell death, playing a deleterious role in the etiology and progression of ischemic stroke [18]. Therefore, following ischemic stroke, how to intervene autophagy development is a key to tissue repair and functional recovery.

Recent studies have shown apoptosis and autophagy share common stimuli and pathways, which creates a complex network between the two pathways [19]. In certain instance, autophagy can block the induction of apoptosis by inhibiting the caspase-dependent pathway, thereby reducing cellular injury [20]. Whereas in other instance, autophagy mechanism leads to increased apoptotic cell death [21]. Autophagy can occur in parallel to apoptosis or the former can be dominant first, yet ultimately leading to autophagy-associated apoptosis [22]. In short, the interplay and crosstalk between autophagy and apoptosis is complex but important.

Salvianolate lyophilized injection (SLI) is the water-soluble extracted from Salvia Miltiorrhiza, and is mainly used for the clinical treatment of mild to moderate cerebral infarction [23]. We previously demonstrated that SLI has significant protection on cerebral I/R injury in rats, which may be related to reduce oxidative stress, suppress inflammatory responses [24]. However, as far as we know, these is no report on how SLI regulates both autophagy and apoptosis, and what is the interplay between them induced by SLI. Therefore, the aim of this study was to explode the effects of SLI on autophagy and apoptosis, and to further investigate the potential molecular mechanisms in the OGD/R-induced N2a cells injury in vitro.

Materials And Methods

Drug and cells culture treatment

SLI, provided by Tianjin Tasly Pharmaceutical Co, Ltd (Tianjin, China), is approved by CFDA (China Food and Drug Administration, GuoYaoZhunZi-Z20110011) in 2011. The fingerprint chromatogram of SLI contains five major bioactive components, namely Sal B, Sal D, Sal Y, alkannic acid and RA [25]. Mouse neuroblastoma N2a cells (ATCC, Manassas, USA) were cultured in Minimum Essential Medium (MEM; Hyclone, USA) containing 10% fetal bovine serum (FBS; Gibco, USA), and antibiotic-antimycotic (Gibco) at 37 °C temperature in a humidified incubator under 5% CO2 and 95% air. The cells were divided into control group, oxygen-glucose deprivation/reoxygenation (OGD/R) group, and SLI groups with different concentration gradients (10, 25, or 50 μg/ml). The OGD/R model was performed as our previously described [26]. N2a cells were cultured in balanced salt solution and then transferred to a special chamber that was closed and included a gas mixture with consisting of 95% N2 and 5% CO2. The special

chamber was placed in an incubator at 37 °C for 4 h. After OGD, N2a cells were maintained in complete medium in a normoxic incubator for 4, 6, or 24 h. Control cells were treated identically without undergoing OGD conditions.

Cell Viability And LDH Release Analysis

Cell viability was analyzed using CCK-8 assay. N2a cell suspension (100 μl/well, 1.0 × 106/ml) was cultured in 96-well microplates until the cells adhered to the wells and grew evenly, which can be used in experiments. After cell grouping treatment, remove the cell supernatant, add 100 μl MEM containing 10% CCK-8 (Dojindo, Japan) into each well of the culture plate, and incubate at 37℃ for 30 min. The absorbance was measured at 450 nm using a microplate reader (TECAN, Switzerland).

Lactate dehydrogenase (LDH) release assay was detected by an LDH Cytotoxicity Assay Kit (Dojindo, Japan). After the cells were incubated in different groups, 25 μl cell supernatant was collected from each well and transferred to the new 96-well microplates and added CytoTox-ONETM Reagent, which were prepared in advance. The new 96-well plate was incubated 20℃ for 30 min, then 50 μl of stop solution was added to the wells, and mixed for 30 s while shaking. The wavelength absorbance at 560/590 nm was measured for the cell viability test (TECAN, Switzerland).

Detection Of Apoptosis By Flow Cytometry

Apoptosis was evaluated by Annexin V-FITC/PI Apoptosis Detection Kit (Invitrogen, USA). After treatment, N2a cells were trypsinized, washed with cold PBS, and then resuspended in 150 μl binding buffer containing 5 μl Annexin-V-FITC and 1 μl propidium iodide (PI). After incubation at 37 °C for 15 min in dark, the fluorescence level was quantified by flow cytometry (BD Biosciences, USA).

Determination Of Cytochrome C Release

Cytochrome c (Cyt-c) release was examined by using the Cytochrome-c ELISA Assay Kit according to manufacturer's instructions (JingKangBio, Shanghai, China). After drug intervention, N2a cells were collected and washed twice. 50 μl of samples and standards were added to the wells of microplate, which were pre-coated with cytochrome c specific antibody. Then 100 μl of a streptavidin-HRP conjugate is added to all wells and incubated at 37 °C for 60 min. Washed with washing buffer and added chromogenic agent A (50 μl) and B (50 μl) into the microwells, and then incubated at 37 °C in the dark for 15 min. After adding stop solution (50 μl), the absorbance was measured using a microplate reader (TECAN, Switzerland).

Western Blotting Analysis

The cells were lysed in cell lysis buffer (Beyotime, Shanghai, China). After centrifugation of 12000 g for 10 min, the supernatant was obtained and used for western blot analysis. Detection of protein concentration was measured using BCA assay kit (Beyotime, Shanghai, China). Subsequently, protein was separated on 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After that, the membrane was incubated with primary antibodies against Cleaved-Caspsase-3 (CST, 9664), Bcl-2 (Abcam, ab27795), LC3B (Abcam, ab48394), Beclin-1 (CST, 3738), p62 (Abcam, ab56416), p-Akt (CST, 4060), Akt (CST, 4685), mTOR (CST, 2983), β-actin (CST, 4970) at 4 °C overnight. After the last wash, the membranes incubated with goat anti-mouse or anti-rabbit peroxidase-conjugated secondary antibody (ZSGB-Bio, Beijing, China) for 1 h. Then, immunoreactive proteins were visualized using chemiluminescence reagent (ECL; Millipore, USA). The staining intensity of each band was evaluated by densitometry and quantified using ImageJ software.

Statistical analysis

The data were analyzed by SPSS 23.0 statistical software, which were expressed as mean ± S.D. The comparison between different groups was analyzed by one-way analysis of variance, followed by Bonferroni's multiple comparison tests. The differences were statistically significant at $P < 0.05$.

Results

Effects of SLI on OGD/R-induced loss of cell viability

The effects of SLI on OGD/R-induced cytotoxicity were detected with CCK-8 assay and LDH release. As shown in Fig. 1, treatment of SLI (10, 25, 50 μg/ml) significantly increased cell viability and decreased LDH release against OGD/R-mediated cytotoxicity.

Effects of SLI on OGD/R-induced cellular apoptosis and apoptosis-related proteins

Annexin V-FITC and PI double staining were used to detect apoptosis of N2a cells after OGD4h/R24h. As shown in Fig. 2A, B, apoptotic rate was much higher in OGD/R group than in control group. Treatment with SLI (25 and 50 μg/ml) down-regulated apoptotic rate significantly, suggesting that it possesses anti-apoptotic property. In addition, it was found that the release of Cyt-c was significantly decreased in cells incubated with 50 μg/ml SLI (Fig. 2C).

In order to further evaluate apoptosis following OGD/R, apoptosis-related proteins were detected using western blot. The results revealed that the expression of Cleaved-caspase-3 increased, while anti-apoptotic protein Bcl-2 decreased (Fig. 2D, E) in the OGD/R group compared to the control group. SLI treatment significantly reduced the expression of Cleaved-caspase-3, and increased Bcl-2 compared to OGD/R group.

SLI enhances autophagy following OGD/R-induced N2a cells

To evaluate the effect of SLI on the autophagy, the autophagy related proteins were assessed by western blot. First, we measured the autophagic protein LC3Ⅱ for 4 h OGD followed by 0–24 h reoxygenation. As it can be seen from Fig. 3A, B, across reoxygenation, the LC3Ⅱ level first decreased and then increased compared with that of the control, and the lowest point at 6 h after reoxygenation. Therefore, we further assessed the effect of SLI on the autophagy proteins expression at OGD4h/R6h. As presented in Fig. 3C-F, LC3Ⅱ and Beclin-1 protein expression was significantly reduced, whilst p62 expression was significantly increased in the OGD/R group compared with the control group. After SLI treatment, especially at 50 μg/ml dose, significantly up-regulated expression of LC3Ⅱ and Beclin-1, and down-regulated expression of p62. These results indicated that SLI enhanced the activation of autophagy in N2a cells following OGD/R, and we found that autophagy alteration precedes apoptosis.

SLI inhibites apoptosis on N2a cells after OGD/R by activating autophagy

To investigate whether inhibition of apoptosis process by SLI is associated with an activation of autophagy in OGD/R-induced N2a cells, the autophagy inhibitor 3-MA was used in this part of the experiment. First, we directly observed the cell viability and LDH release of OGD/R-induced N2a cells (Fig. 4A, B). The results suggested that the SLI-induced protection was reversed by 3-MA. Then, we also observed the apoptosis rate and Cyt-c release (Fig. 4C-E). Similar results, apoptosis rate and Cyt-c release showed an enlargement in SLI + 3-MA group than SLI group. Collectively, our results suggested activating autophagy may play a key role in SLI-mediated inhibition of apoptosis.

SLI Regulates Akt/mTOR Signaling Pathway In N2a Cells

The Akt/mTOR pathway has been described as an important autophagy regulatory signaling pathway. Akt can activate its downstream mTOR to inhibit the occurrence of autophagy. Therefore, we examined the phosphprylation of Akt and mTOR level in OGD/R induced N2a cells in vitro. Western blot analysis revealed that both the levels of phosphorylation of Akt and mTOR were increased at OGD4h/R6h. After administration treatment, SLI significantly decreased the ratio of p-Akt/Akt and mTOR/β-actin compared with the OGD/R group (Fig. 5A-C).

Discussion

Ischemic stroke is severe brain disease, which refers to cerebral vascular stenosis or occlusion, ultimately leading to hypoxia or even necrosis of brain tissue [27]. In Chinese traditional medicine, ischemic stroke is considered to belong to the category of blood stasis syndrome, and “huo xue hua yu” is the most common method. Salvia miltiorrhiza is one of the most commonly used drugs for “huo xue hua yu”. Salvianolate lyophilized injection (SLI) is separated from water-soluble extract of Salvia miltiorrhiza by freeze-drying. It has been widely used to treat acute cerebral infarction in clinical in China. We previously reported that SLI can promote the recovery of nerve function post stroke rats in MCAO/R or diabetic model [28, 29]. The mechanism may be related to reduce oxidative stress [29], repress inflammatory responses [30] and strengthen BBB function [31]. However, the underlying molecular mechanism is not

comprehensively clear and warrants further investigation. In this study, we detected the protection mechanism of SLI in vitro using an OGD/R model in N2a cells. And we demonstrated that SLI confers an extraordinary neuro-protection against OGD/R-induced N2a cells disruption and the mechanism be verified that reducing neuronal apoptosis by activating Akt/mTOR dependent autophagy pathway.

Increasing evidence confirms that apoptosis is an important mechanism of secondary injury, which increases significantly during brain I/R injury [32–34]. During apoptosis, Cyt-c is released from the mitochondria intermembrane space into the cytosol. Subsequently recruiting and resulting in cascade reaction of caspase. Ultimately, caspase-3 efficiently cleaves, leading to apoptosis [35]. Bcl-2 family mainly involved in mitochondrial mediated apoptosis pathway [36]. Bcl-2 is an important anti-apoptotic protein expressed in surviving neurons. The protein bcl-2 can exert its antiapoptotic effect by binding to adenosine translocation factor which competes with Bax. Our results showed that SLI significantly reduced apoptosis rate by flow cytometry, inhibited the release of Cyt-c, decreased Cleaved-caspase-3 expression, and increased Bcl-2 level in OGD/R N2a cells (Fig. 2). Evidence suggested that the SLI served as an anti-apoptosis element in vitro. However, there are many questions remain to be further elucidated, especially regarding the role and the underlying mechanisms of SLI in the anti-apoptosis effect of ischemic stroke.

Autophagy is a dynamic process that decomposes the damaged organelles, abnormal proteins, and metabolic waste and then maintains the normal physiological microenvironment [37]. It is worth noting that autophagy is considered to be the self-protection mechanism of cells, but in some cases, autophagy may promote cellular demise through excessive self-digestion or stimulation of activation of other cell death pathways [38]. Recently, increasing evidence has supported that regulating autophagy is a mechanism of cerebral I/R injury [39–41]. Luo et al. found that inhibition of autophagy via activation of PI3K/Akt pathway contributes to against neuronal death caused by ischemic insults [42]. In contrast, Yu's group also suggested that moderate activation of autophagy can increase autophagy of hippocampal neurons by regulating mTOR, reduce reperfusion injury, and protect neurons [43]. Actually, autophagy is a double-edged sword [44]. Under certain conditions, moderate autophagy can elimination defective organelles and maintain the normal cellular homeostasis and cell vitality [45]. However, long-term excessive autophagy brings redundant degradation of cell contents, leading to cell injury and apoptosis [46]. There is conflicting report as to the role of autophagy in ischemic stroke, which may be related to the degree of damage and the effect time. Therefore, whether autophagy is beneficial or detrimental in ischemic brain remains to be further discussed. In this experiment, we found that the level of autophagy first decreased and then increased with increasing duration of reoxygenation. And it will restore to control level until reoxygenation for 24 h. That is, autophagy is impaired in early stage of cerebral ischemia, in keeping with previous reports [47]. In the present our experiment, we found that treatment with SLI during hypoxia-reoxygenation can abrogate OGD/R-induced autophagy inhibition, increased the expression of LC3Ⅱ and Beclin-1, decrease the accumulation of p62, and ultimately ameliorate cell viability reduction. In short, SLI showed obvious protection of auto-phagocytosis in vitro.

Autophagy and apoptosis are two important processes of self-destruction, which interact with each other in cell death and survival [48]. Emerging evidence demonstrated that many stress pathways sequentially elicit autophagy and apoptosis within the nerve cells. The interplay between them is also complex but important. Luo et al. confirmed that upregulated autophagy may present as an anti-apoptotic mechanism by eliminating of apoptosis-associated molecules [49]. However, He et al. studies suggested that apoptosis activation is often related to increased autophagy processes [50]. In many cases autophagy precedes apoptosis and several proteins (e.g. BECN1) play important roles in both pathways [51, 52]. Sirois et al. observed that autophagy predated development of apoptosis and persisted while apoptosis was activated [53]. In our present study, the autophagy inhibition was at its lowest point at reoxygenation 6 h, before rising gradually. And apoptosis rate was much higher than that of the control group at reoxygenation 24 h. It is indicated that autophagy precedes apoptosis occur, which is consistent with literatures. The limitation of this study is no time-dependent analysis of apoptosis occurs, and autophagy detection only persisted to reoxygenation 24 h. Whether autophagy will continue to rise and lead to excessive autophagy is unclear. However, in Fig. 2, 3, it is clear that SLI able to reverse OGD/R-induced autophagy inhibition and occurrence of apoptosis. And the time of activating autophagy is earlier than that of SLI inhibiting apoptosis. As expected, when autophagy was suppressed by 3-MA, an autophagy inhibitor, the protection capacity of SLI on cells viability, apoptosis, and Cyt-c leakage were attenuated, suggesting that SLI inhibits cell apoptosis by up-regulating autophagy (Fig. 4).

The Class PI3K/Akt/mTOR signaling pathway is a well-known autophagy pathway [54]. mTOR is a major negative regulator of autophagy and it is downstream to the PI3K/Akt pathway [55]. It has been recently reported that the neuroprotective effect of neurotrophic factor, is mediated by autophagy through the PI3K/Akt/mTOR pathway in cortical neurons [56]. Meanwhile, PI3K/Akt/mTOR pathway is a key intracellular signaling transduction pathway that drives cellular growth and represses cell apoptosis [57]. The Akt/mTOR pathway maybe associated with both autophagy and apoptosis and possible common upstream pathway [58, 59]. The inhibition of the PI3K/Akt/mTOR signal transduction pathway is consistently associated with triggering autophagy and reducing neuronal apoptosis [60]. Based on this pathway, we observed the p-Akt and mTOR expressions in N2a cells treated by OGD/R with or without SLI in vitro (Fig. 5). The results revealed that SLI can down-regulated the expressions of p-Akt and mTOR, thereby promoting autophagy. This indicated that the neuroprotective effect of SLI on ischemic stroke can be explained by regulating the autophagic activity via Akt/mTOR pathway. Of course, it should be pointed out that the Akt/mTOR signal pathway is not the only regulator of also modulate the autophagy. To further clarify whether SLI could regulate the Akt pathway, Ly294002, an Akt phosphorylation inhibitor, should be used in future research.

In this study, we focused on the relationship between autophagy and apoptosis after OGD/R induced N2a cells and investigated the role of the Akt/mTOR signaling pathway in the regulation of them. Our study demonstrated that SLI can induce autophagy and suppress apoptosis after OGD/R injury, at least in part, through Akt/mTOR-dependent pathway.

Conclusions

The present study indicated that SLI has potential as a novel therapy for ischemic stroke, and its possible mechanism is to induced autophagy and inhibit apoptosis through blocking Akt/mTOR signaling pathway in OGD/R-induced N2a cells, providing new insights into the mechanism of SLI in the treatment of ischemic stroke.

List Of Abbreviations

3-MA,3-methyladenine; BBB, blood-brain barrier; Bcl-2, B-cell lymphoma-2; BSCB, blood-spinal cord barrier; CFDA, China Food and Drug Administration; CM, Chinese medicine; DLA, diastereomer of lithospermic acid; DSal E, diastereomer of salvianolic acid E; ER, endoplasmic reticulum; HPLC, High Performance Liquid Chromatograph; I/R, ischemia/reperfusion; LA, lithospermic acid; LDH, Lactate dehydrogenase; MCAO/R, Middle Cerebral Artery occlusion/ reperfusion; OGD, oxygen and glucose deprivation; OGD/R, oxygen-glucose deprivation/reoxygenation; PI, propidium iodide; PRO, Protocatechualdehyde; PVDF, polyvinylidene difluoride; RA, rosmarinic acid; Sal B, salvianolic acid B; Sal D, salvianolic acid D; Sal E, salvianolic acid E; Sal Y, salvianolic acid Y.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The manuscript is approved by all authors for publication.

Availability of data and materials

The data and materials generated or analyzed during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author contribution statement

Dongna Li: Conceptualization, Methodology, Writing - Original Draft

Mengmeng Ma: Analyzed data, Performed research

Wenqi Zhang: Contributed new reagents or analytic tools

Yangyang Xu: Analyzed data

Shaoxia Wang; Lijuan Chai: Supervision, Validation

Hong Guo; Limin Hu: Writing- Reviewing and Editing

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References

1. Feigin, V. L., Norrving, B., George, M. G., Foltz, J. L., Roth, G. A., & Mensah, G. A. (2016) Prevention of stroke: a strategic global imperative. Nature Reviews Neurology, 12(9), 501.
2. Ramos, E., Patiño, P., Reiter, R. J., Gil-Martín, E., Marco-Contelles, J., Parada, E., ... & Egea, J. (2017) Ischemic brain injury: new insights on the protective role of melatonin. Free Radical Biology and Medicine, 104, 32-53.
3. Dirnagl, U., Iadecola, C., & Moskowitz, M. A. (1999) Pathobiology of ischaemic stroke: an integrated view. Trends in neurosciences, 22(9), 391-397.
4. Lo, E. H., Dalkara, T., & Moskowitz, M. A. (2003) Mechanisms, challenges and opportunities in stroke. Nature reviews neuroscience, 4(5), 399-414.
5. Eltzschig, H. K., & Eckle, T. (2011) Ischemia and reperfusion—from mechanism to translation. Nature medicine, 17(11), 1391.
6. Chen, S. D., Wu, C. L., Hwang, W. C., & Yang, D. I. (2017) More insight into BDNF against neurodegeneration: anti-apoptosis, anti-oxidation, and suppression of autophagy. International journal of molecular sciences, 18(3), 545.
7. Wang, P., Shao, B. Z., Deng, Z., Chen, S., Yue, Z., & Miao, C. Y. (2018) Autophagy in ischemic stroke. Progress in neurobiology, 163, 98-117.
8. Valdor, R., & Macian, F. (2012) Autophagy and the regulation of the immune response. Pharmacological research, 66(6), 475-483.
9. Doria Andrea., Gatto Mariele., Punzi Leonardo (2013) Autophagy in human health and disease. N. Engl. J. Med., 368(19), 1845.
10. Bar-Yosef, T., Damri, O., & Agam, G. (2019) Dual role of autophagy in diseases of the central nervous system. Frontiers in cellular neuroscience, 13.
11. Long, M., & McWilliams, T. G. (2019) Monitoring autophagy in cancer: From bench to bedside. In Seminars in cancer biology. Academic Press.

12. Zhang, X., Yan, H., Yuan, Y., Gao, J., Shen, Z., Cheng, Y., ... & Wang, G. (2013) Cerebral ischemia-reperfusion-induced autophagy protects against neuronal injury by mitochondrial clearance. Autophagy, 9(9), 1321-1333.
13. Feng, J., Chen, X., & Shen, J. (2017) Reactive nitrogen species as therapeutic targets for autophagy: implication for ischemic stroke. Expert opinion on therapeutic targets, 21(3), 305-317.
14. Wang, P., Shao, B. Z., Deng, Z., Chen, S., Yue, Z., & Miao, C. Y. (2018). Autophagy in ischemic stroke. Progress in Neurobiology, S0301008217301442.
15. Yameng, S., Ting, Z., Yan, Z., Jinfeng, L., Lei, J., & Yinyi, S., et al. (2018). Ischemic postconditioning alleviates cerebral ischemia–reperfusion injury through activating autophagy during early reperfusion in rats. Neurochemical Research.
16. Tu, Y., Guo, C., Song, F., Huo, Y., & Fan, W.. (2019). Mild hypothermia alleviates diabetes aggravated cerebral ischemic injury via activating autophagy and inhibiting pyroptosis. Brain Research Bulletin, 150.
17. Chen, W. , Sun, Y. , Liu, K. , & Sun, X. . (2014). Autophagy: a double-edged sword for neuronal survival after cerebral ischemia. Neural Regen Res, 9(12), 1210-6.
18. Mo, Y. , Sun, Y. Y. , & Liu, K. Y. . (2020). Autophagy and inflammation in ischemic stroke. Neural Regeneration Research, 15(8), 1388.
19. Mukhopadhyay, S., Panda, P. K., Sinha, N., Das, D. N., & Bhutia, S. K. (2014) Autophagy and apoptosis: where do they meet?. Apoptosis, 19(4), 555-566.
20. Li, Z. R., Ma, T., Guo, Y. J., Hu, B., Niu, S. H., Suo, F. Z., ... & Mamun, M. A. A. (2019) Sanggenon O induced apoptosis of A549 cells is counterbalanced by protective autophagy. Bioorganic Chemistry, 87, 688-698.
21. Ho Wing-Mann., Akyol Onat., Reis Haley., Reis Cesar., McBride Devin., Thome Claudius., Zhang John. (2018). Autophagy after Subarachnoid Hemorrhage: Can Cell Death be Good? Curr Neuropharmacol, 16(9), 1314-1319.
22. Lange Carsten., Lehmann Christiane., Mahler Martin., Bednarski Patrick J.(2019). Comparison of Cellular Death Pathways after mTHPC-mediated Photodynamic Therapy (PDT) in Five Human Cancer Cell Lines. Cancers (Basel), 11(5), undefined.
23. Zhou, L., Zuo, Z., & Chow, M. S. S. (2005) Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. The Journal of Clinical Pharmacology, 45(12), 1345-1359.
24. Wang, F. J., Wang, S. X., Chai, L. J., Zhang, Y., Guo, H., & Hu, L. M. (2018) Xueshuantong injection (lyophilized) combined with salvianolate lyophilized injection protects against focal cerebral ischemia/reperfusion injury in rats through attenuation of oxidative stress. Acta Pharmacologica Sinica, 39(6), 998-1011.
25. Zhuang, P., Wan, Y., Geng, S., He, Y., Feng, B., Ye, Z., ... & Zhang, Y. (2017) Salvianolic Acids for Injection (SAFI) suppresses inflammatory responses in activated microglia to attenuate brain damage in focal cerebral ischemia. Journal of ethnopharmacology, 198, 194-204.

26. Chai, L., Guo, H., Li, H., Wang, S., Wang, Y. L., Shi, F., ... & Adah, D. (2013) Scutellarin and caffeic acid ester fraction, active components of Dengzhanxixin injection, upregulate neurotrophins synthesis and release in hypoxia/reoxygenation rat astrocytes. Journal of ethnopharmacology, 150(1), 100-107.
27. Hankey, G. J. (2017) Stroke. Lancet, 389(10069), 641-654.
28. He, Q., Wang, S., Liu, X., Guo, H., Yang, H., Zhang, L., ... & Hu, L. (2015) Salvianolate lyophilized injection promotes post-stroke functional recovery via the activation of VEGF and BDNF-TrkB-CREB signaling pathway. International journal of clinical and experimental medicine, 8(1), 108.
29. Wang, F. J., Wang, S. X., Chai, L. J., Zhang, Y., Guo, H., & Hu, L. M. (2018) Xueshuantong injection (lyophilized) combined with salvianolate lyophilized injection protects against focal cerebral ischemia/reperfusion injury in rats through attenuation of oxidative stress. Acta Pharmacologica Sinica, 39(6), 998-1011.
30. Wang, F., He, Q., Wang, J., Yuan, Q., Guo, H., Chai, L., ... & Zhang, Y. (2017) Neuroprotective effect of salvianolate lyophilized injection against cerebral ischemia in type 1 diabetic rats. BMC complementary and alternative medicine, 17(1), 258.
31. Zhao, C., Li, X., Li, X., Xu, Y., Ma, M., Wang, S., ... & Hu, L. (2019) Salvianolate lyophilized injection (SLI) strengthens blood-brain barrier function related to ERK1/2 and Akt signaling pathways. Brain research, 1720, 146295.
32. Gong, L., Tang, Y., An, R., Lin, M., Chen, L., & Du, J. (2017) RTN1-C mediates cerebral ischemia/reperfusion injury via ER stress and mitochondria-associated apoptosis pathways. Cell death & disease, 8(10), e3080-e3080.
33. Deng, Y., Ma, G., Dong, Q., Sun, X., Liu, L., Miao, Z., & Gao, F. (2019) Overexpression of miR-224-3p alleviates apoptosis from cerebral ischemia reperfusion injury by targeting FIP200. Journal of cellular biochemistry, 120(10), 17151-17158.
34. Tian, Z., Tang, C., & Wang, Z. (2019) Neuroprotective effect of ginkgetin in experimental cerebral ischemia/reperfusion via apoptosis inhibition and PI3K/Akt/mTOR signaling pathway activation. Journal of cellular biochemistry, 120(10), 18487-18495.
35. Guo, Y., Zhao, Y., Zhou, Y., Tang, X., Li, Z., & Wang, X. (2019) LZ-101, a novel derivative of danofloxacin, induces mitochondrial apoptosis by stabilizing FOXO3a via blocking autophagy flux in NSCLC cells. Cell death & disease, 10(7), 1-14.
36. Belzacq, A. S., Vieira, H. L., Kroemer, G., & Brenner, C. (2002) The adenine nucleotide translocator in apoptosis. Biochimie, 84(2-3), 167-176.
37. Wollert, T., 2019. Autophagy. Current Biology, 29(14), R671-R677.
38. Kasprowska-Liśkiewicz, D. (2017) The cell on the edge of life and death: Crosstalk between autophagy and apoptosis. Postepy higieny i medycyny doswiadczalnej (Online), 71, 825-841.
39. He, Q., Li, Z., Wang, Y., Hou, Y., Li, L., & Zhao, J. (2017) Resveratrol alleviates cerebral ischemia/reperfusion injury in rats by inhibiting NLRP3 inflammasome activation through Sirt1-dependent autophagy induction. International immunopharmacology, 50, 208-215.

40. Huang, X., Ding, J., Li, Y., Liu, W., Ji, J., Wang, H., & Wang, X. (2018) Exosomes derived from PEDF modified adipose-derived mesenchymal stem cells ameliorate cerebral ischemia-reperfusion injury by regulation of autophagy and apoptosis. Experimental cell research, 371(1), 269-277.
41. Zhang, Y., Zhang, Y., Jin, X. F., Zhou, X. H., Dong, X. H., Yu, W. T., & Gao, W. J. (2019) The role of astragaloside IV against cerebral ischemia/reperfusion injury: suppression of apoptosis via promotion of P62-LC3-autophagy. Molecules, 24(9), 1838.
42. Luo, T., Liu, G., Ma, H., Lu, B., Xu, H., Wang, Y., ... & Liang, J. (2014) Inhibition of autophagy via activation of PI3K/Akt pathway contributes to the protection of ginsenoside Rb1 against neuronal death caused by ischemic insults. International journal of molecular sciences, 15(9), 15426-15442.
43. Yu, B., Ruan, M., Liang, T., Huang, S. W., Yu, Y., Cheng, H. B., & Shen, X. C. (2017) The synergic effect of tetramethylpyrazine phosphate and borneol for protecting against ischemia injury in cortex and hippocampus regions by modulating apoptosis and autophagy. Journal of Molecular Neuroscience, 63(1), 70-83.
44. Wei Kai., Wang Pei., Miao Chao-Yu. (2012) A double-edged sword with therapeutic potential: an updated role of autophagy in ischemic cerebral injury. CNS Neurosci Ther, 18(11), 879-86.
45. Hou Kai., Xu Dan., Li Fengyang., Chen Shijie., Li Yunman. (2019) The progress of neuronal autophagy in cerebral ischemia stroke: Mechanisms, roles and research methods. J. Neurol. Sci., 400(undefined), 72-82.
46. Chung Hyunju., Choi Junghyun., Park Seungjoon. (2018) Ghrelin protects adult rat hippocampal neural stem cells from excessive autophagy during oxygen-glucose deprivation. Endocr. J., 65(1), 63-73.
47. Wang Pei., Guan Yun-Feng., Du Hui., Zhai Qi-Wei., Su Ding-Feng., Miao Chao-Yu. (2012) Induction of autophagy contributes to the neuroprotection of nicotinamide phosphoribosyltransferase in cerebral ischemia. Autophagy, 8(1), 77-87.
48. Gordy, C., & He, Y. W. (2012) The crosstalk between autophagy and apoptosis: where does this lead?. Protein & cell, 3(1), 17-27.
49. Luo, S., & Rubinsztein, D. C. (2013) BCL2L11/BIM: a novel molecular link between autophagy and apoptosis. Autophagy, 9(1), 104-105.
50. He Guoqian., Xu Wenming., Tong Linyan., Li Shuaishuai., Su Shiceng., Tan Xiaodan., Li Changqing. (2016) Gadd45b prevents autophagy and apoptosis against rat cerebral neuron oxygen-glucose deprivation/reperfusion injury. Apoptosis, 21(4), 390-403.
51. Young Christopher N J., Sinadinos Anthony., Lefebvre Alexis., Chan Philippe., Arkle Stephen., Vaudry David., Gorecki Dariusz C. (2015) A novel mechanism of autophagic cell death in dystrophic muscle regulated by P2RX7 receptor large-pore formation and HSP90. Autophagy, 11(1), 113-30.
52. Franzetti Eleonora., Huang Zhi-Jun., Shi Yan-Xia., Xie Kun., Deng Xiao-Juan., Li Jian-Ping., Li Qing-Rong., Yang Wan-Ying., Zeng Wen-Nian., Casartelli Morena., Deng Hui-Min., Cappellozza Silvia., Grimaldi Annalisa., Xia Qingyou., Feng Qili., Cao Yang., Tettamanti Gianluca. (2012) Autophagy precedes apoptosis during the remodeling of silkworm larval midgut. Apoptosis, 17(3), 305-24.

53. Sirois Isabelle., Groleau Jessika., Pallet Nicolas., Brassard Nathalie., Hamelin Katia., Londono Irène., Pshezhetsky Alexey V., Bendayan Moise., Hébert Marie-Josée. (2012) Caspase activation regulates the extracellular export of autophagic vacuoles. Autophagy, 8(6), 927-37.
54. Heras-Sandoval, D., Pérez-Rojas, J. M., Hernández-Damián, J., & Pedraza-Chaverri, J. (2014) The role of PI3K/AKT/mTOR pathway in the modulation of autophagy and the clearance of protein aggregates in neurodegeneration. Cellular signalling, 26(12), 2694-2701.
55. Jung, C. H., Ro, S. H., Cao, J., Otto, N. M., & Kim, D. H. (2010) mTOR regulation of autophagy. FEBS letters, 584(7), 1287-1295.
56. Bak Dong Ho., Zhang Enji., Yi Min-Hee., Kim Do-Kyung., Lim Kyu., Kim Jwa-Jin., Kim Dong Woon. (2015) High ω3-polyunsaturated fatty acids in fat-1 mice prevent streptozotocin-induced Purkinje cell degeneration through BDNF-mediated autophagy. Sci Rep, 5(undefined), 15465.
57. Sun Dongying., Lei Wei., Hou Xiaodong., Li Hui., Ni Wenlu. (2019) PUF60 accelerates the progression of breast cancer through downregulation of PTEN expression. Cancer Manag Res, 11(undefined), 821-830.
58. Trejo-Solís Cristina., Serrano-Garcia Norma., Escamilla-Ramírez Ángel., Castillo-Rodríguez Rosa A., Jimenez-Farfan Dolores., Palencia Guadalupe., Calvillo Minerva., Alvarez-Lemus Mayra A., Flores-Nájera Athenea., Cruz-Salgado Arturo., Sotelo Julio. (2018) Autophagic and Apoptotic Pathways as Targets for Chemotherapy in Glioblastoma. Int J Mol Sci, 19(12), undefined.
59. Trejo-Solís Cristina., Serrano-Garcia Norma., Escamilla-Ramírez Ángel., Castillo-Rodríguez Rosa A., Jimenez-Farfan Dolores., Palencia Guadalupe., Calvillo Minerva., Alvarez-Lemus Mayra A., Flores-Nájera Athenea., Cruz-Salgado Arturo., Sotelo Julio. (2018) Autophagic and Apoptotic Pathways as Targets for Chemotherapy in Glioblastoma. Int J Mol Sci, 19(12), undefined.
60. Wang ZhenYu., Zhou LinQuan., Zheng XiaoTing., Chen Gang., Pan RongFeng., Li JinHuang., Liu WenGe. (2017) Autophagy protects against PI3K/Akt/mTOR-mediated apoptosis of spinal cord neurons after mechanical injury. Neurosci. Lett., 656(undefined), 158-164.

Figures

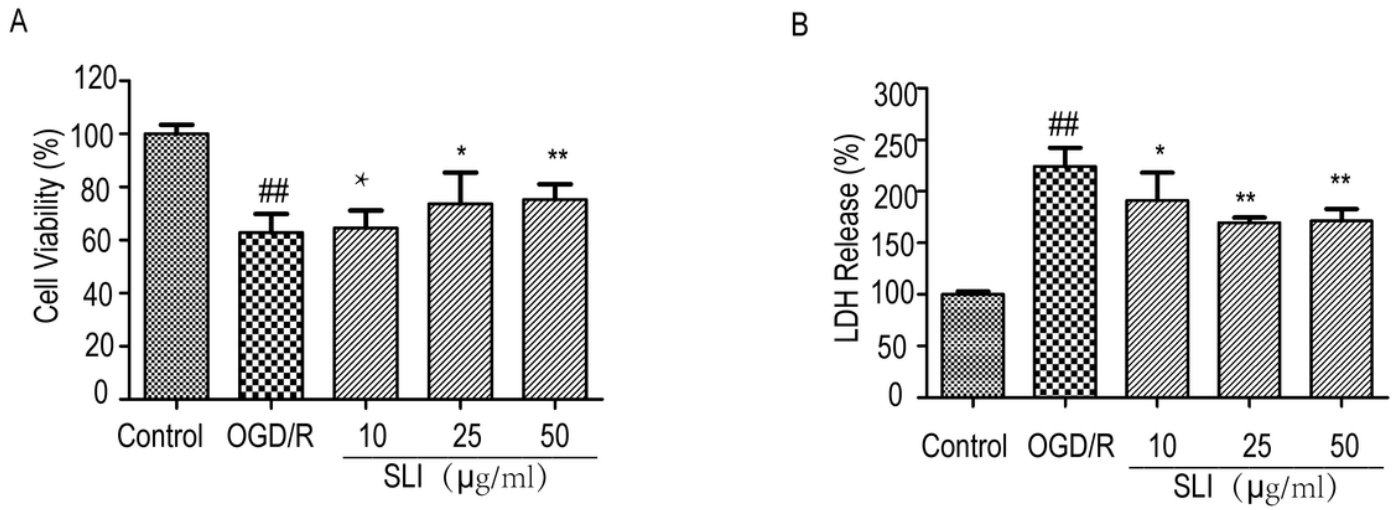

Figure 1

SLI rescued OGD/R-induced loss of cell viability. N2a cells were incubated with different concentrations of SLI (10, 25, 50μg/ml) during the process of OGD/R. Cellular viability was detected with CCK-8 (A) and LDH release (B) analysis. Data shown are the mean±SD. ##P<0.01 vs. Control group, *P<0.05, **P<0.01 vs. OGD/R group, n=6.

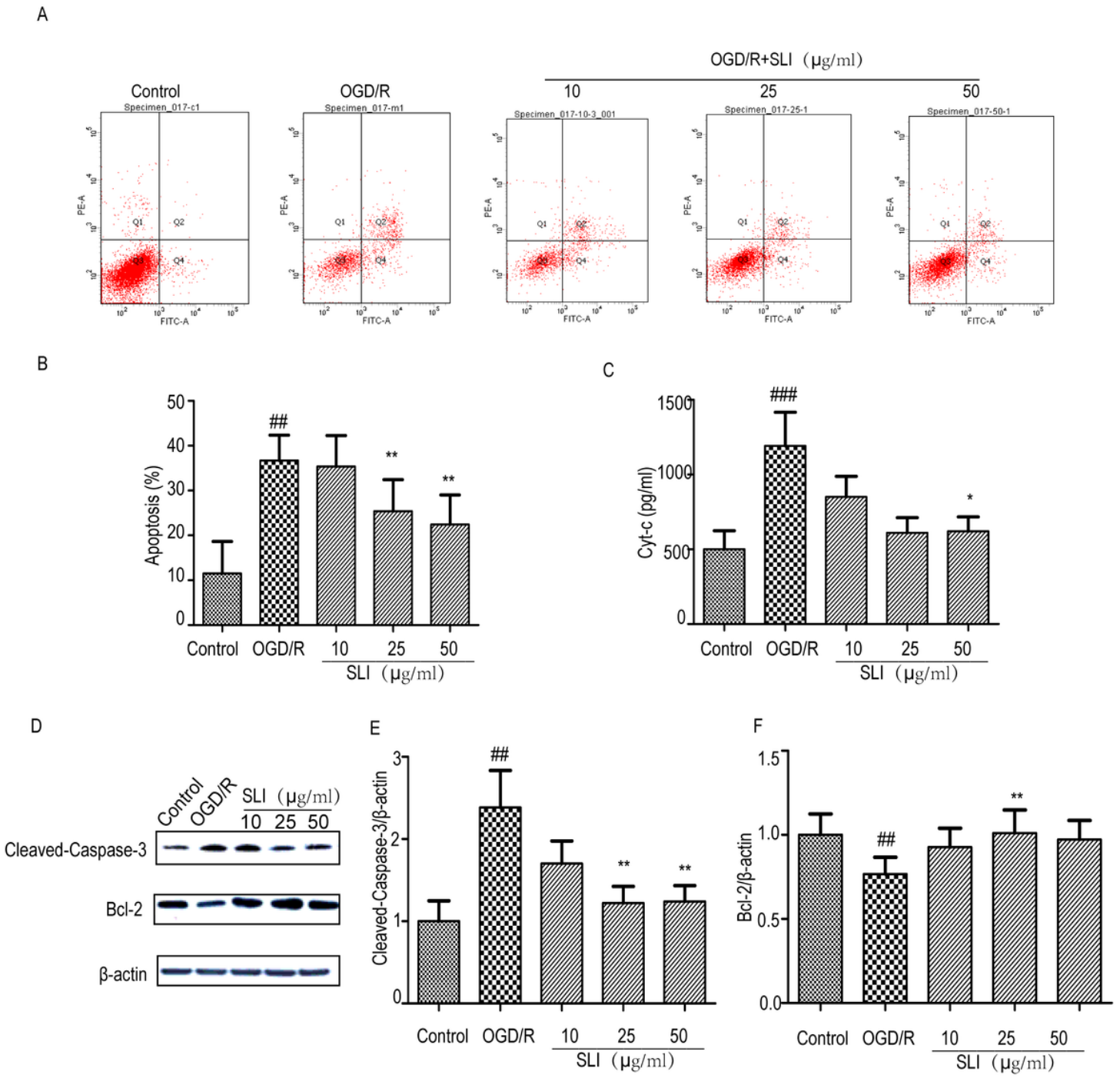

Figure 2

SLI inhibited the neuronal apoptosis induced by OGD/R. After treated with different concentrations of SLI (10, 25, 50μg/ml) during OGD/R, cells were collected and detected by flow cytometry, Cytc-c assay, and western blot. (A) Flow cytometry plots showed that SLI prevented OGD/R-induced neuronal apoptosis, as shown by the decrease in the percentage of apoptotic cells in comparison to OGD/R condition. (B) Column bar graph of apoptotic rate. (C) The release of Cyt-C from the mitochondria in N2a cells. (D) The representative immunoblot bands. The quantification histogram of (E) Cleaved-caspase-3 and (F) Bcl-2 to

β-actin. Data shown are the mean±SD. ##P<0.01, ###P<0.001 vs. Control group, *P<0.05, **P<0.01 vs. OGD/R group, n=3.

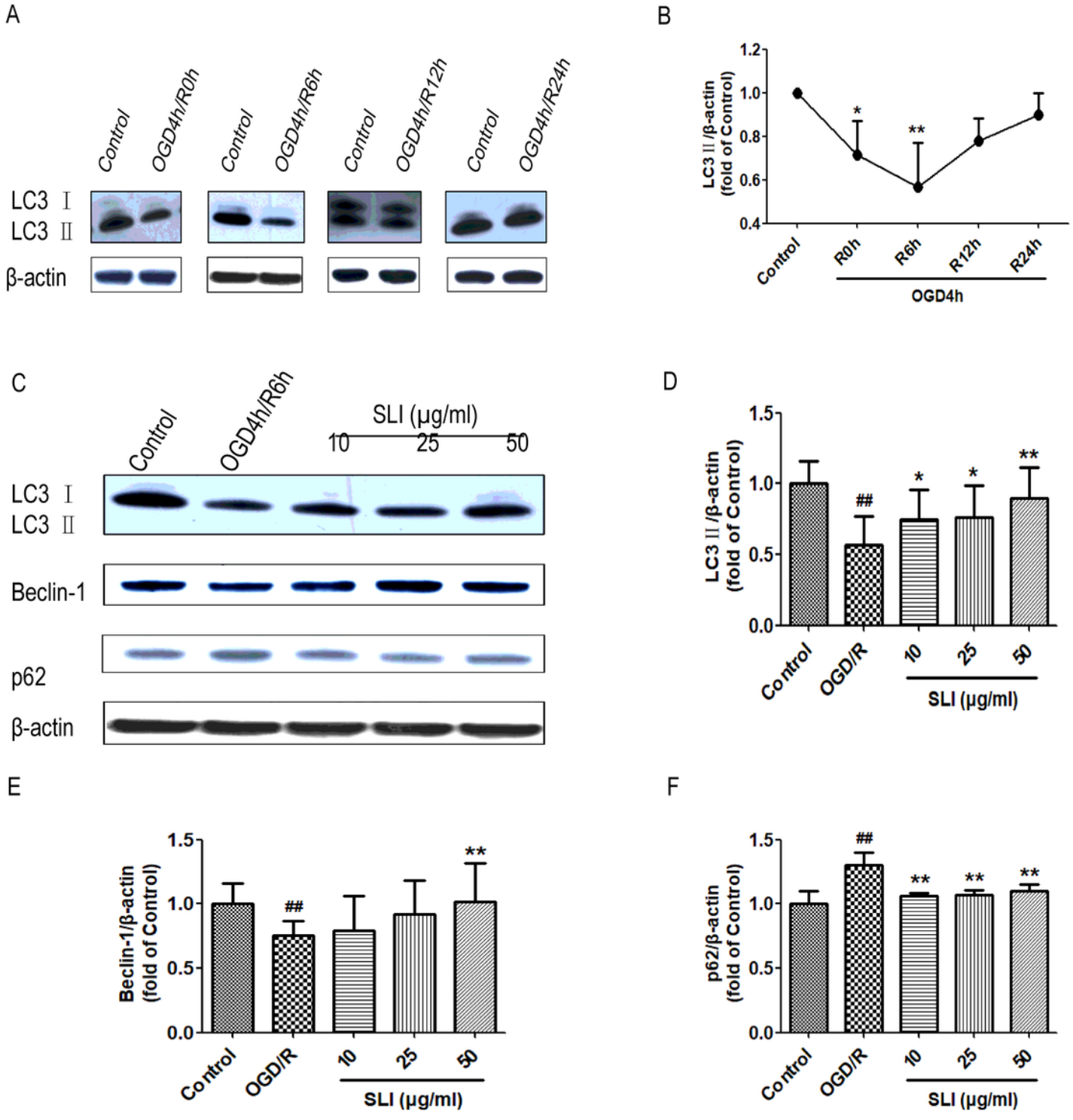

Figure 3

SLI enhanced autophagy in OGD/R-induced N2a cells. N2a cells were treated with OGD 4h and reoxygenation 0-24h. (A) The changes of LC3Ⅱ level across reoxygenation 0-24h. (B) Line chart of LC3Ⅱ change. N2a cells were treated for OGD4h/R6h with or without co-treatment with SLI, and cells were

collected and detected by western blot. (C) The representative immunoblot bands. The quantification histogram of (D) LC3Ⅱ, (E) Beclin-1, and (F) p62 to β-actin. Data shown are the mean±SD. #P<0.05, ##P<0.01 vs. Control group, *P<0.05, **P < 0.01 vs. OGD/R group, n=3.

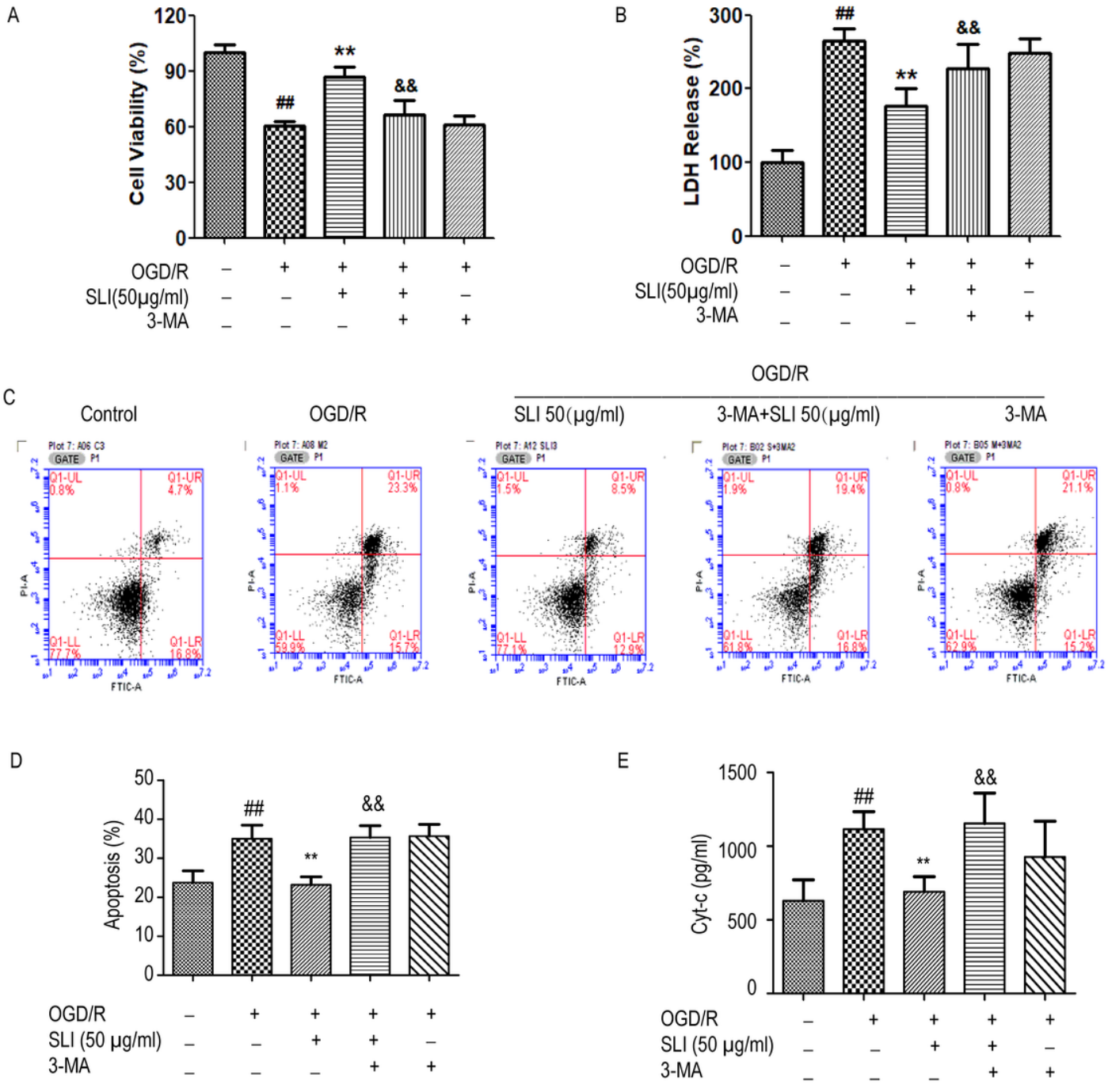

Figure 4

Autophagy inhibitor antagonized SLI-suppressed apoptosis in N2a cells. N2a cells were pretreated with 2.5μM autophagy inhibitor 3-MA for 1h before administration of SLI. Pre-incubation of 3-MA blocked the effect of SLI to increase (A) cell viability and repress (B) LDH release. (C, D)The 3-MA significantly

increased apoptosis rate and inhibited the effect of SLI on apoptosis rate. (E) Pre-incubation of 3-MA attenuated the effect of SLI to decrease the release of Cyt-c. Data shown are the mean±SD. ##P<0.01 vs. Control group, **P<0.01 vs. OGD/R group, &&P<0.01 vs. SLI group, n=3.

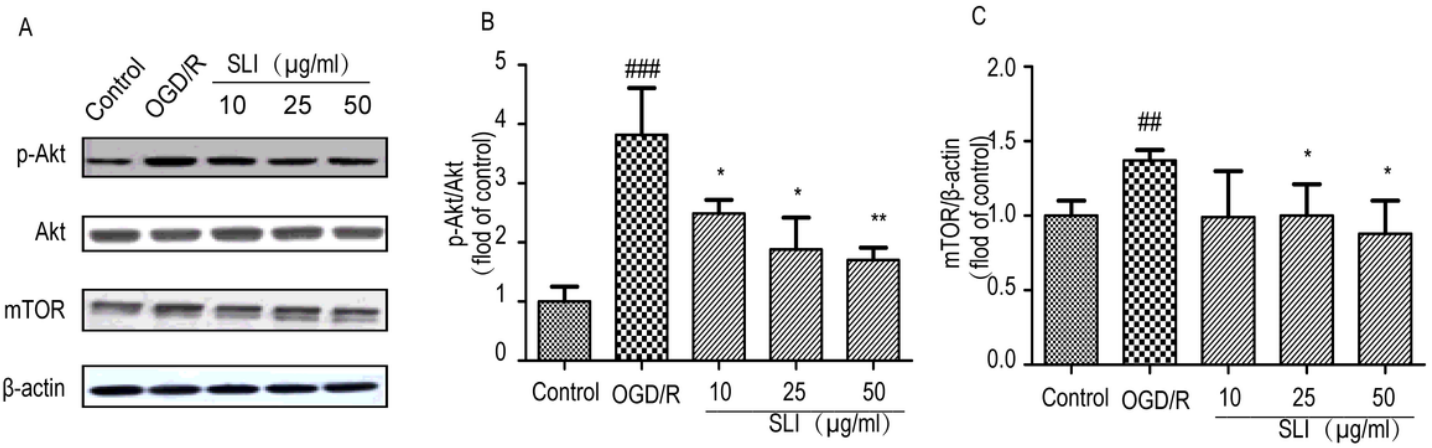

Figure 5

SLI regulates Akt/mTOR signaling pathway in N2a cells. N2a cells were cultured for OGD4h/R6h in the absence and presence of SLI (10, 25, 50μg/ml). After that, the cells were collected and detected by western blot. (A) The representative immunoblot bands of p-Akt, Akt, mTOR, and β-actin. SLI (10, 25 and 50μg/ml) significantly decreased the ratio of (B) p-Akt/Akt and (C) mTOR/β-actin compared with the OGD/R group. Data shown are the mean±SD. ##P<0.01, ###P<0.001 vs. Control group, *P<0.05, **P<0.01 vs. OGD/R group, n=3.

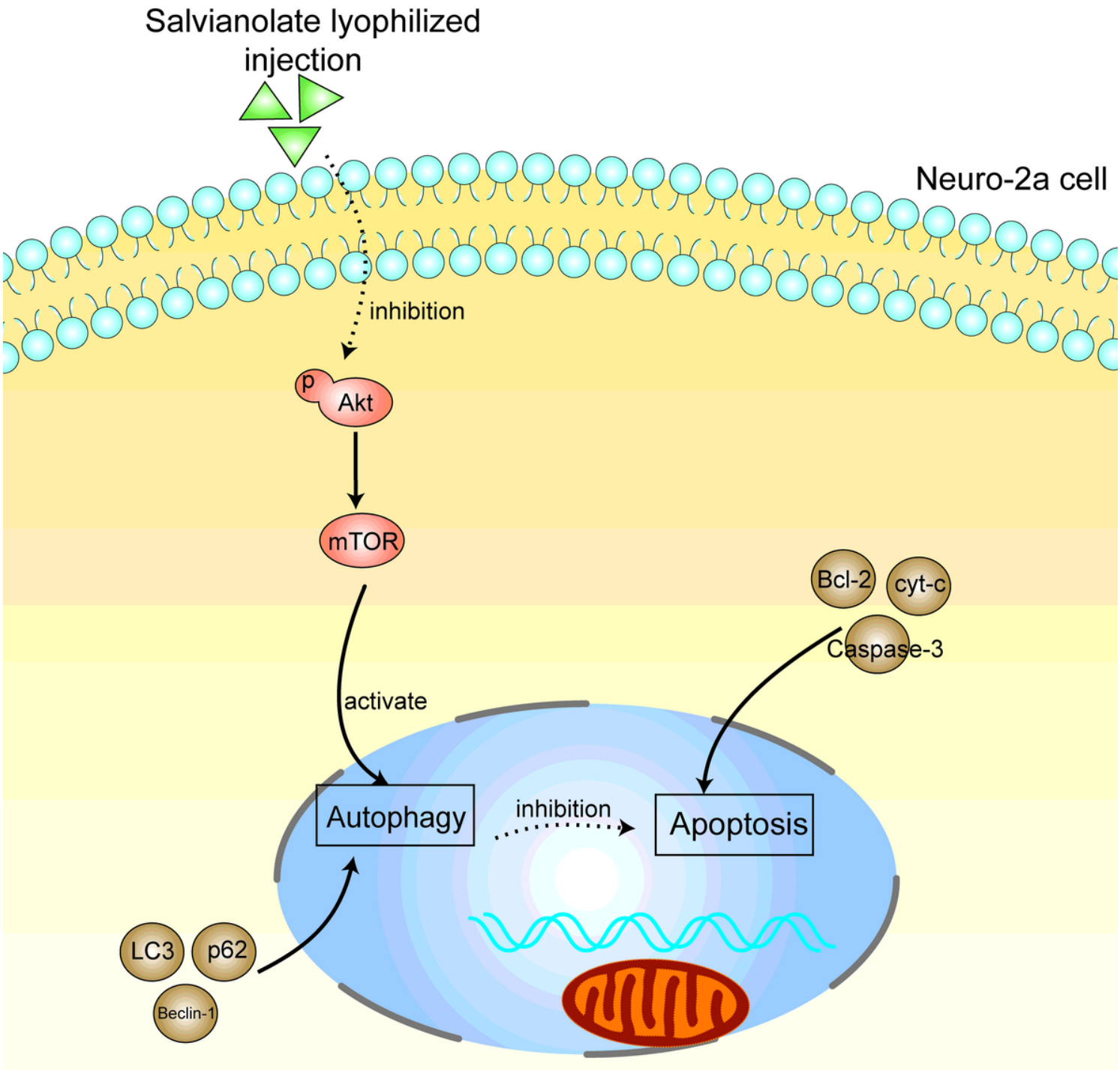

Figure 6

The neuroprotective mechanism of SLI attenuates apoptosis through Akt/mTOR dependent autophagy activation. SLI down-regulated Akt/mTOR may trigger autophagy. Autophagy is activated in response to SLI neuroprotection. SLI induced autophagy can further inhibit apoptosis. There is considerable crosstalk among autophagy and apoptosis.