



Research Article

Stem-leaf saponins from *Panax notoginseng* counteract aberrant autophagy and apoptosis in hippocampal neurons of mice with cognitive impairment induced by sleep deprivation

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ABSTRACT

Background: Sleep deprivation (SD) impairs learning and memory by inhibiting hippocampal functioning at molecular and cellular levels. Abnormal autophagy and apoptosis are closely associated with neurodegeneration in the central nervous system. This study is aimed to explore the alleviative effect and the underlying molecular mechanism of stem–leaf saponins of *Panax notoginseng* (SLSP) on the abnormal neuronal autophagy and apoptosis in hippocampus of mice with impaired learning and memory induced by SD.

Methods: Mouse spatial learning and memory were assessed by Morris water maze test. Neuronal morphological changes were observed by Nissl staining. Autophagosome formation was examined by transmission electron microscopy, immunofluorescent staining, acridine orange staining, and transient transfection of the tf-LC3 plasmid. Apoptotic event was analyzed by flow cytometry after PI/annexin V staining. The expression or activation of autophagy and apoptosis-related proteins were detected by Western blotting assay.

Results: SLSP was shown to improve the spatial learning and memory of mice after SD for 48 h, accompanied with restrained excessive autophagy and apoptosis, whereas enhanced activation of phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin signaling pathway in hippocampal neurons. Meanwhile, it improved the aberrant autophagy and apoptosis induced by rapamycin and re-activated phosphoinositide 3-kinase/Akt/mammalian target of rapamycin signaling transduction in HT-22 cells, a hippocampal neuronal cell line.

Conclusion: SLSP could alleviate cognitive impairment induced by SD, which was achieved probably through suppressing the abnormal autophagy and apoptosis of hippocampal neurons. The findings may contribute to the clinical application of SLSP in the prevention or therapy of neurological disorders associated with SD.

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Sleep plays an important role in the central nervous system restoration [1], memory consolidation [2–5], and affect regulation [6,7]. Sleep deprivation (SD), including absent, insufficient, and fragmented sleep, is prevalent in modern societies and may cause comprehensive neurobehavioral and physiological consequences [6,8,9]. Numerous studies indicated that SD is closely associated with many psychiatric and neurodegenerative disorders, such as schizophrenia, depression, anxiety, substance abuse disorder,

Alzheimer's disease, Parkinson's disease, and multiple sclerosis [6,10]. Although the underlying pathophysiological mechanism of SD-induced cognitive impairment is still largely elusive [11], insufficient sleep has been shown to produce molecular- and cellular-level alterations that profoundly inhibit hippocampal functioning [8].

Autophagy is an evolutionarily conserved pathway, which controls the degradation of the superfluous or dysfunctional proteins

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and organelles [12]. It plays a crucial role in maintaining the physiological processes, such as neurodevelopment and neurogenesis, of the central nervous system [13]. However, stressors, such as hypoxia, cellular stress, nutrient starvation, and reactive oxygen species [14,15], may induce excessive autophagy that causes neuronal atrophy [16] and programmed cell death [17]. Apoptosis is a physiological program of cellular death, which contributes to the neuronal cell loss in many neurological disorders [18]. Intimate connections have been implicated between autophagy and apoptosis because both the processes share some of the same regulators [19]. Sleep fragmentation has been shown to alter circadian rhythm of autophagy proteins in the hippocampus [20]; nevertheless, whether SD induces excessive autophagy connected with apoptosis in hippocampal neurons has not been elucidated.

Panax notoginseng (Burk.) F.H.Chen is a perennial herb of *Panax* genus in the Araliaceae family, the rhizome of which is traditionally used for the therapy of blood diseases. Saponins extracted from the rhizome of *P. notoginseng* are taken as the major active constituents and widely used to treat stroke, cerebral ischemia, and cardiovascular diseases [21–24]. Owing to the difference of the main structure, saponins from *P. notoginseng* are classified into protopanaxadiol (PPD) and protopanaxatriol type [25]. Stem–leaf saponins of *P. notoginseng* (SLSP) are different from those of the underground parts in terms of compositions and contents. SLSP mainly compose of plentiful PPD-type saponins, such as ginsenoside Rb3, Rb1, Rc, and notoginsenoside Fc [26–28]. 20(S)-PPD has been demonstrated to alleviate oxidative stress and improve cognitive impairment induced by chronic SD in mice [25]. However, whether SLSP have similar pharmacological functions as 20(s)-PPD has not been clarified. Moreover, whether SLSP can alleviate excessive autophagy and apoptosis of hippocampal neurons also has not been elucidated. Therefore, the present study was aimed to investigate the effect of SLSP on cognitive impairment and autophagy and apoptosis of hippocampal neurons of mice induced by SD, which may facilitate the clinical application of SLSP.

1. Materials and methods

1.1. Reagents

SLSP was purchased from Qidan Co. Ltd. (purity > 95%, Wenshan, Yunnan, China). The main saponins of SLSP were ginsenoside Rb1 (4.86%), ginsenoside Rc (11.1%), notoginsenoside Fc (11.8%), ginsenoside Rb3 (17.4%), notoginsenoside FP2 (5.59%), notoginsenoside Fa (4.13%), ginsenoside Rd (3.04%), notoginsenoside IX (7.72%), notoginsenoside Fe (5.45%), and gypenoside XVII (3.67%).

Antibodies against Beclin-1 (Cat. No. 3495, Rabbit mAb, 1:1000), LC3B (3868, Rabbit mAb, 1:1000), phospho–phosphoinositide 3-kinase (PI3K) p85 (4228, Rabbit mAb, 1:1000), phospho-Akt (9271, Rabbit mAb, 1:1000), PI3K p85 (4257, Rabbit mAb, 1:1000), Akt (9272, Rabbit mAb, 1:1000), Bcl-2 (2870, Rabbit mAb, 1:1000), Bax (2772, Rabbit mAb, 1:1000), and cleaved caspase-3 (9664, Rabbit mAb, 1:1000) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against p62 (ab109012, Rabbit mAb, 1:1000), p-mTOR (ab84400, Rabbit mAb, 1:1000), mTOR (ab87540, Rabbit mAb, 1:1000), and β -actin (ab8227, Rabbit mAb, 1:1000) were purchased from Abcam (Cambridge, MA, USA). Rapamycin and 3-methyladenine (3-MA) were provided by Selleckchem (Boston, MA, USA). Akt inhibitor VIII were bought from EMD Chemicals (An Affiliate of Merck KGaA, Darmstadt, Germany). All the other reagents were of analytical grade unless mentioned otherwise.

1.2. Animals and treatment

Male C57BL/6 mice, 5 weeks old, were provided by Animal Research Center of Shanghai University of Traditional Chinese Medicine (Shanghai, China). All mice were housed under the controlled environment ($23 \pm 2^\circ\text{C}$, 12-h light/dark cycle) supplied with food and water *ad libitum*. Animals were allowed to habituate the environment for 1 week before the experiments. All animal studies were carried out complying with the Institutional Animal Care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine.

The mice were randomly divided into six groups, which were named as control group, SD group, modafinil (13 mg/kg) group, SLSP 25 mg/kg group, SLSP 50 mg/kg group, and SLSP 100 mg/kg group ($n = 10/\text{group}$). Animals in control and SD groups were intragastrically given distilled water while those in the modafinil group and SLSP groups were intragastrically administered with respective drugs consecutively for 9 days.

1.3. Morris water maze test

The learning and memory behaviors of mice were examined by Morris water maze test as described previously [29]. The timeline of the experiment was shown in Fig. 1. Mice in the SD group, modafinil group, and SLSP groups were sleep deprived for 48 h after the hidden platform test according to a modified multiple platform method [30]. In brief, groups of six animals were placed in polypropylene cages ($41 \times 34 \times 16$ cm). Each cage contained 12 platforms (3-cm diameter and 5-cm height) submerged into water up to 1 cm below the surface of the platforms. The animals could move

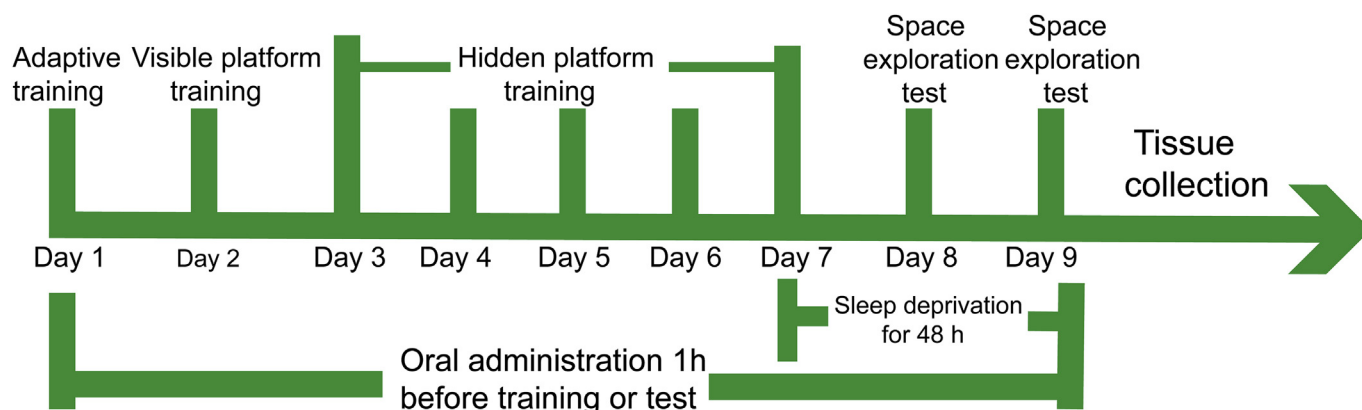


Fig. 1. Timeline for the behavioral test.

freely, jumping from one platform to another one. Food and water were available during the entire procedure. The space exploration tests were conducted after SD for 24 h and 48 h, respectively, to evaluate the retention of spatial memory in the tests. The mice were allowed to freely swim for 60 s in the water maze without the platform. The frequencies of an individual mouse passing through the platform area and the detention time in the target quadrant were recorded as a measurement of spatial memory. The movement track of mice was monitored with a computerized tracking system. On the completion of the last space exploration test, the mice were sacrificed with excessive 1% pentobarbital sodium.

1.4. Transmission electron microscopy

After transcardially perfused with phosphate buffered saline (PBS), the mice were fixed with 2% glutaraldehyde. CA3 region of the mouse hippocampus was diced ($0.5 \times 0.5 \times 1.0$ mm) and sectioned into ultrathin slices, which were further stained with uranyl acetate and lead citrate after dehydration. The ultrastructural images of CA3 region were taken under H-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

1.5. Immunohistochemistry

After anesthetized with excessive 1% pentobarbital sodium, the mice were perfused intracardially with PBS followed by 4% paraformaldehyde. Brains of mice were dissected and fixed in 4% paraformaldehyde at 4 °C overnight. Then they were sequentially dehydrated with 15% and 30% sucrose solution, embedded with embedding medium (Sakura Finetek, USA, 3801480) and cyrosectioned into 20 μ m-thick slices. After permeabilized and blocked with 5% donkey serum in PBS containing 0.3% Triton X-100 for half an hour, the sections were incubated with primary antibodies against LC3B at 4 °C overnight followed by secondary antibodies conjugated with Alexa fluorophore. Fluorescent pictures were taken with Olympus VS120 Virtual Slide Scanner (Olympus).

1.6. Nissl staining

The brain sections were hydrated with gradient alcohol (95%, 80%, and 70%). After being washed three times with distilled water, they were stained with 1% Toluidine blue reagent preheated to 60°C for 40 min. Then, they were washed three times with distilled water and differentiated with 95% ethanol until the

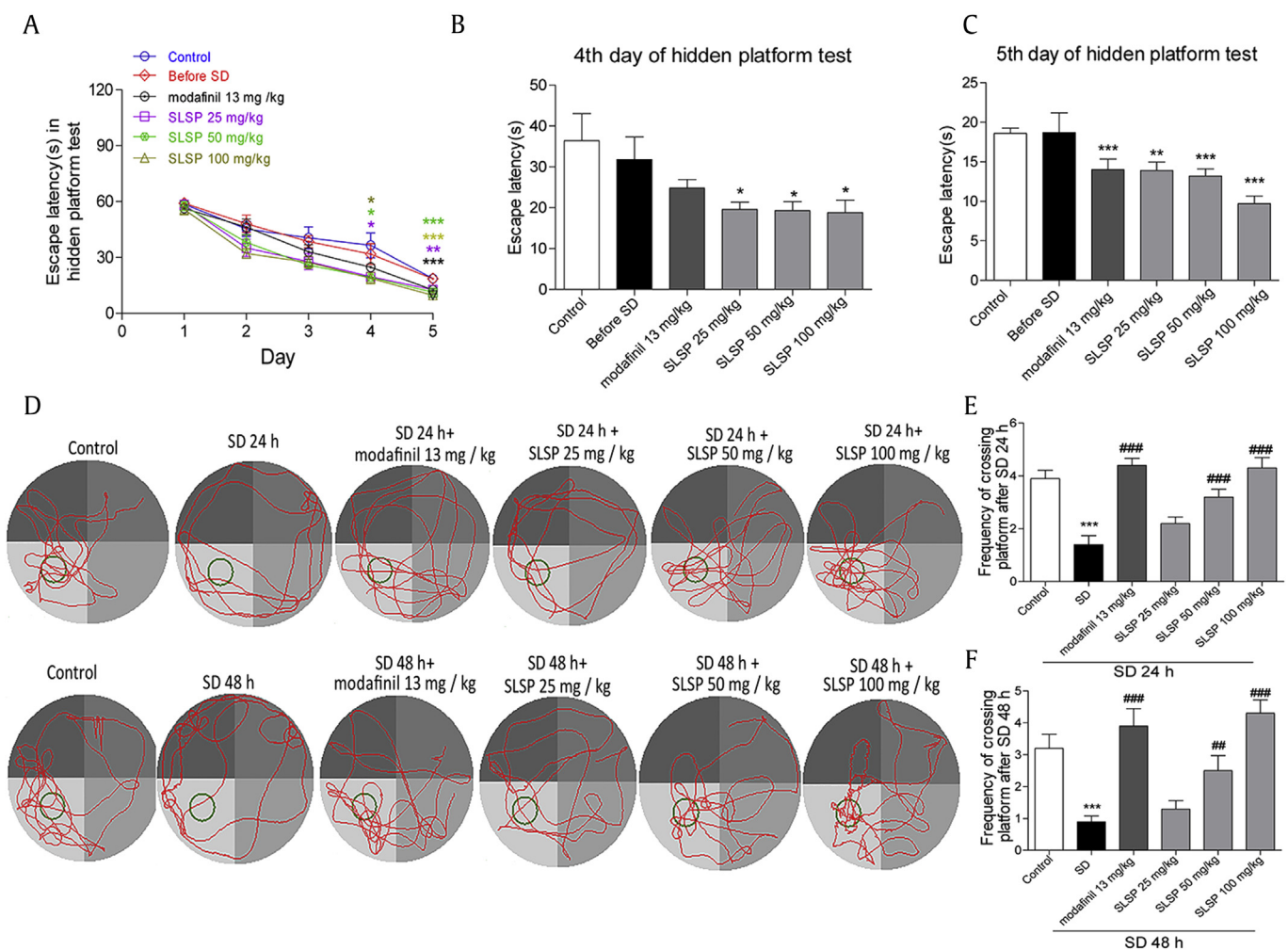


Fig. 2. The effect of SLSP on learning and memory of SD mice tested by Morris water maze. (A) Escape latency of mice in hidden platform test for 5 consecutive days. (B) Escape latency of mice in hidden platform test of 4th day. (C) Escape latency of mice in hidden platform test of 5th day. (D) Representative swimming tracks of mice after SD. (E) Frequency of crossing the platform of mice after 24-h sleep deprivation. (F) Frequency of crossing the platform of mice after 48-h sleep deprivation. N = 10/group. One-way ANOVA with Dunnett *post hoc* analysis was carried out to analyze the differences among groups using GraphPad Prism 5.0. ANOVA, analysis of variance; SD, sleep deprivation; SLSP, stem-leaf saponins of *Panax notoginseng*. *P < 0.05; **P < 0.01; ***P < 0.0001 vs control group. ##P < 0.01; ###P < 0.0001 vs. SD group.

background was clear. Finally, the slides were cleared with xylene and sealed with neutral gum. The staining pictures were taken with Olympus VS120 Virtual Slide Scanner. Compared with the normal cells with regular shape and evenly stained, the injured neuronal cells were shrunken and sickle shaped and stained darkly with fragmented or no nucleus. The percentage of the injured cells within the CA3 region was calculated to evaluate the effect of SD on the structural damage of hippocampal neurons. For each mouse, one of the representative coronal brain sections with the similar coordinate (bregma, -2.0 mm) was used for the statistical purpose.

1.7. Cell culture and treatment

Mouse hippocampal cell line HT-22 was provided by Guangzhou Jennio Biotech Company (Guangzhou, China) and cultured in high-glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). After growing to

semiconfluence, the cells were pretreated with rapamycin ($50 \mu\text{g/ml}$) for 4 h to induce autophagy, followed by the treatment of 3-MA (2.5 mmol/L) or SLSP ($12.5, 25, 50 \mu\text{g/ml}$) for 48 h. Finally, the cells were harvested for further analysis.

1.8. Plasmid transfection

The tf-LC3 plasmid was kindly provided by Dr. Cheng Huang (Drug Discovery Lab, School of Pharmacy, Shanghai University of Traditional Chinese Medicine) and transfected into HT-22 cells using Lipofectamine 2000 according to the protocol of the manufacturer. In brief, HT-22 cells (1×10^5 cells/well) cultured in 24-well plate were transiently transfected with the tf-LC3 plasmid ($0.5 \mu\text{g/well}$) in optmedium for 6 h. Then, the cells were induced with rapamycin ($50 \mu\text{g/ml}$) for 4 h followed by the treatment of 3-MA (2.5 mmol/L) or SLSP ($12.5, 25, 50 \mu\text{g/ml}$) for 48 h. Fluorescent pictures of the cells were taken with an inverted fluorescence microscope.

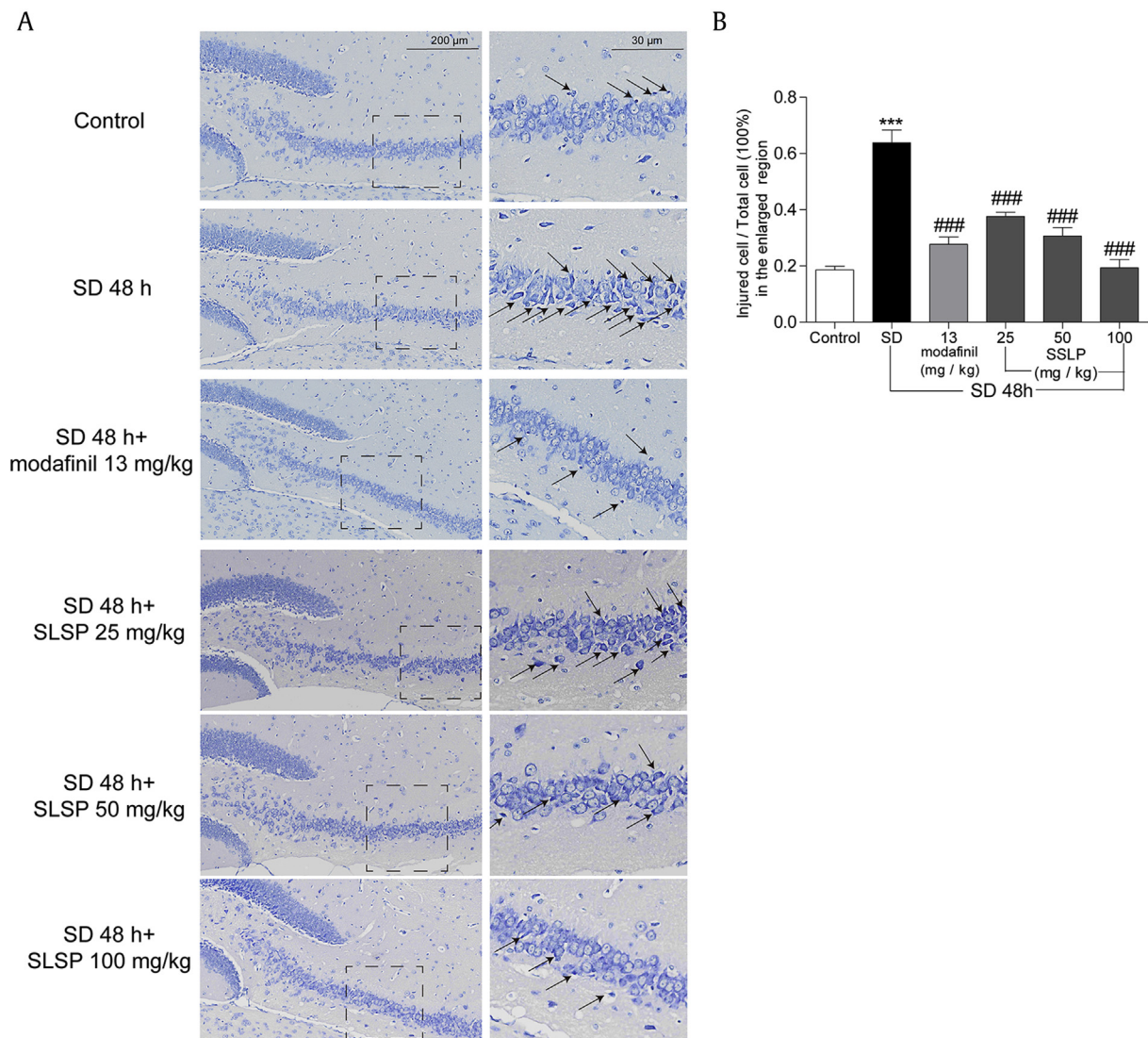


Fig. 3. The effect of SLSP on the hippocampal neuronal morphology of SD mice. (A) Nissl's staining of the dentate gyrus and CA3 of the hippocampus. The pictures in the right panel were the enlarged regions in the rectangular boxes in the left panel, respectively. (B) The percentage of injured cells in the enlarged region of hippocampus. $N = 3/\text{group}$. One-way ANOVA with Dunnett *post hoc* analysis was carried out to analyze the differences among groups using GraphPad Prism 5.0. ANOVA, analysis of variance; SD, sleep deprivation; SLSP, stem-leaf saponins of *Panax notoginseng*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ vs control group. ### $P < 0.01$; #### $P < 0.0001$ vs. SD group.

1.9. Acridine orange staining

HT-22 cells (1×10^5 cells/well) were cultured in 24-well plates and induced with rapamycin (50 $\mu\text{g}/\text{ml}$) for 4 h. Then, they were treated with SLSP (12.5, 25, 50 $\mu\text{g}/\text{ml}$) or 3-MA (2.5 mmol/L) for 48 h. After being washed twice with PBS, the cells were stained with acridine orange (10 $\mu\text{g}/\text{ml}$) for 15 min at 37 °C in the dark. The fluorescent pictures were taken with an inverted fluorescence microscope to assess the autophagy of HT-22 cells.

1.10. Annexin V/PI staining

After being induced with rapamycin (50 $\mu\text{g}/\text{ml}$) for 4 h, HT-22 cells were treated with SLSP (12.5, 25, 50 $\mu\text{g}/\text{ml}$) or 3-MA (2.5 mmol/L) for 48 h. Then, the cells were harvested by trypsinization and washed twice with PBS. Thereafter, they were stained with PI and annexin V according to the protocol of the kit. The apoptosis rate was detected on a flow cytometer (Guava easyCyte HT, Millipore, Germany).

1.11. Western blotting analysis

Protein samples were prepared and quantified as described previously [31]. Totally, 20-mg proteins from each sample were loaded and separated by dodecyl sulfate sodium salt (SDS)-polyacrylamide gel electrophoresis (PAGE) (10% or 12%). After being transferred onto poly vinylidene fluoride (PVDF) membranes and blocked with 5% nonfat milk solution, the protein blots were incubated with respective primary antibodies overnight at 4 °C. Thereafter, the membranes were washed with $1 \times$ PBST (PBS containing 0.1% Tween 20) and incubated with respective secondary

antibodies for 1 h at room temperature. The protein bands were visualized by ECL-prime kit and quantified with Image J 1.46r software (NIH, USA).

1.12. Statistical analysis

All data were presented as mean \pm standard error of the mean. One-way analysis of variance with Dunnett *post hoc* analysis was carried out to analyze the differences among groups using GraphPad Prism 5.0. P-values less than 0.05 were regarded as statistically significant.

2. Results

2.1. SLSP alleviated the cognitive impairments of SD mice

Before SD, all mice were trained in the Morris water maze for the equivalent spatial learning and memory assessment. As shown in Fig. 2A, the escape latency of all mice in the hidden platform test was gradually decreased with the increase of training time, suggesting the good learning capacity of the mice. However, both modafinil and SLSP could enhance the spatial learning and memory of mice. On Day 4 and Day 5 of the hidden platform test (Fig. 2A–2C), compared with the control group mice, modafinil- and SLSP-treated mice spent less time to find the hidden platform ($P < 0.01$ or $P < 0.001$). SD impaired the spatial memory of mice strikingly. As shown in Fig. 2D–2F, after SD for 24 h or 48 h, the mice in the SD group passed through the platform less times in contrast to those in the control group ($P < 0.05$, $P < 0.01$ or $P < 0.001$). Modafinil treatment significantly attenuated the impaired spatial memory of mice ($P < 0.001$). SLSP treatment also improved the behavior of SD mice

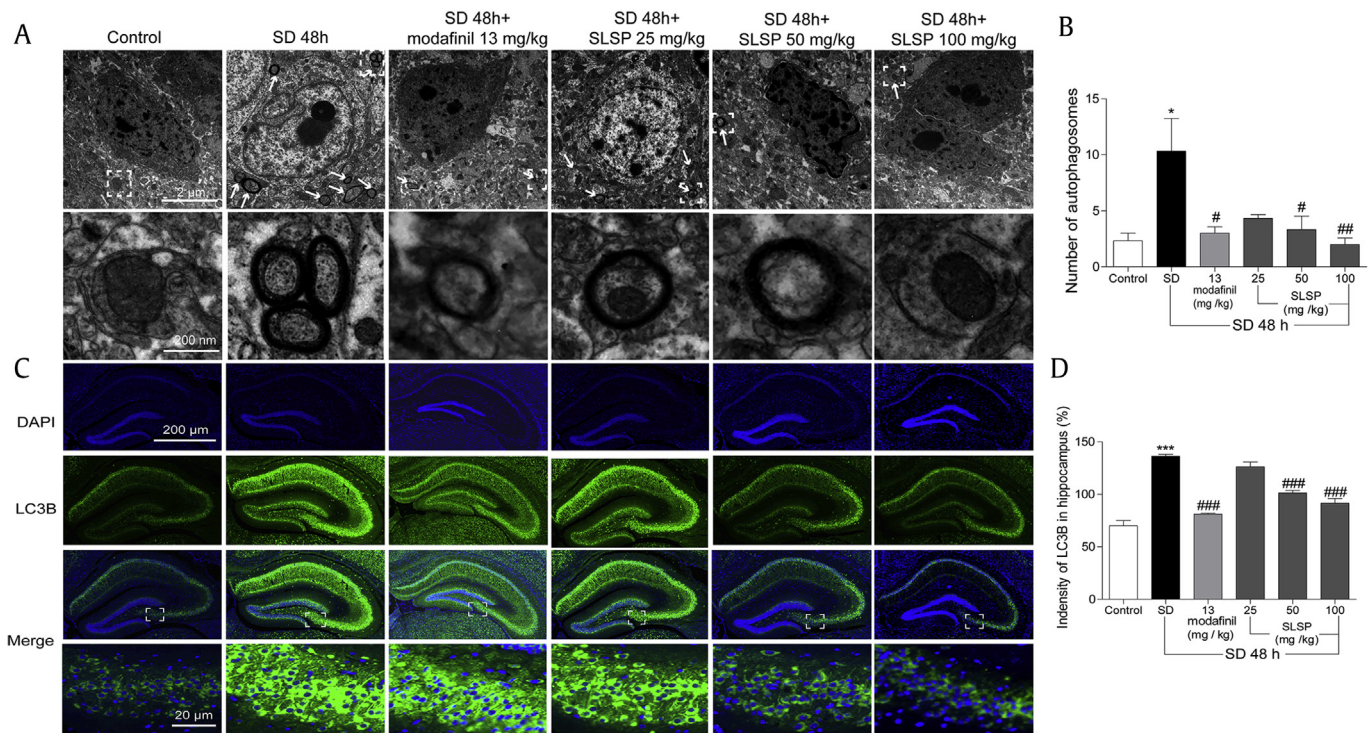


Fig. 4. The effect of SLSP on the formation of autophagosomes and the expression of LC3B in the hippocampus of SD mice. (A) Images of hippocampus of mice obtained from transmission electron microscopy. The arrows denote the autophagosomes. The pictures in the lower panel were the enlarged regions in the rectangular boxes in the upper panel, respectively. (B) The number of autophagosomes. (C) The expression of LC3B in hippocampus of mice. (D) Quantification of LC3B intensities in hippocampus. $N = 3/\text{group}$. One-way ANOVA with Dunnett *post hoc* analysis was carried out to analyze the differences among groups using GraphPad Prism 5.0. ANOVA, analysis of variance; SD, sleep deprivation; SLSP, stem-leaf saponins of *Panax notoginseng*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ vs control group. ## $P < 0.01$; ### $P < 0.0001$ vs. SD group.

and seemed to be in a dose-dependent manner. As displayed in Fig. 2E, 2F, compared with the mice in the SD group, the mice treated with SLSP (50 and 100 mg/kg) passed through the target quadrant more times after SD for 24 h and 48 h ($P < 0.01$ or $P < 0.001$).

2.2. SLSP impeded the morphological change of hippocampal neurons in SD mice

SD changed the morphology of cell bodies of neurons in the cornu ammonis (CA) and dentate gyrus (DG) regions of the mouse hippocampus. As shown in Fig. 3A, neurons in the hippocampal CA3 and DG regions of normal mice were well organized and densely packed. The cell bodies of neurons were plump, and the nucleus was large and round. However, in the SD group mice, the morphology of neurons in the hippocampal CA3 and DG regions of mice was injured. The cell bodies were atrophied with darker Nissl staining. Both modafinil and SLSP treatment (25, 50, 100 mg/kg) reduced the morphological changes significantly in these regions. Most of the hippocampal neuronal cells in mice of modafinil and SLSP treatment groups were evenly stained light blue and had

regularly shaped cell bodies. Correspondingly, the percentage of injured cells in the CA3 region of SLSP-treated group mice (50 and 100 mg/kg) was significantly reduced (Fig. 3B, $P < 0.01$) compared with that in the SD model group. These results implicated that SLSP could prevent neuronal damage in hippocampal regions of mice induced by SD.

2.3. SLSP suppressed the neuronal autophagy in hippocampus of SD mice

To explore if SLSP affected the hippocampal neuronal autophagy of SD mice, the ultrathin brain sections were subjected to transmission electron microscopy observation. As displayed in Fig. 4A, SD induced markedly accumulated autophagosomes with double membranes in hippocampal neurons. SLSP could prominently reduce the number of autophagosomes. To confirm the anti-autophagic effect of SLSP, brain sections of mice were next subjected to LC3B immunostaining. As exposed in Fig. 4B, in CA and DG regions of SD mice, the expression of LC3B in neurons was enhanced markedly compared with that in the control group. SLSP,

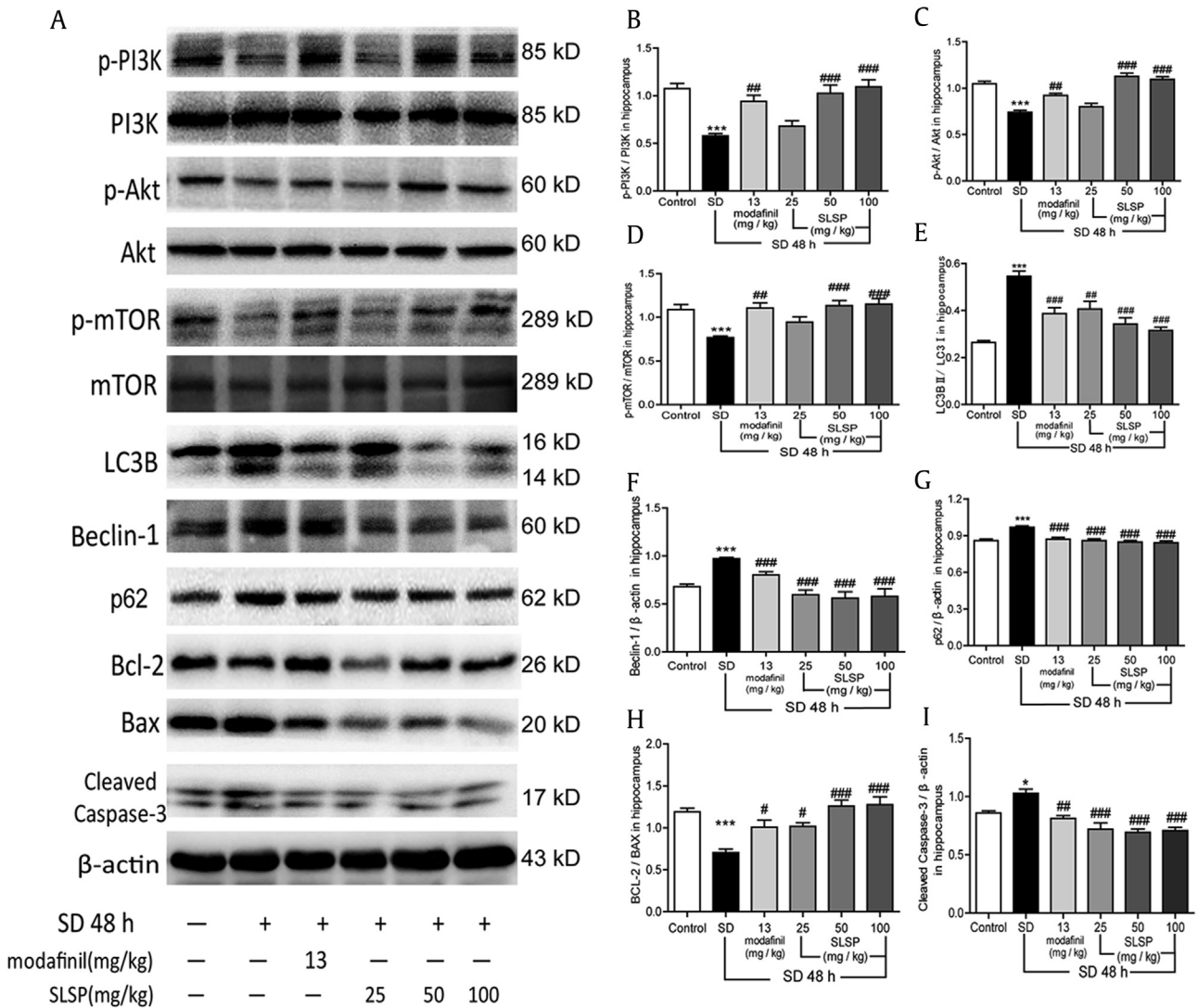


Fig. 5. The effect of SLSP on the proteins related with apoptosis and autophagy in the hippocampus of SD mice. (A) Western blotting bands of respective proteins in hippocampus of mice. (B)-(I) Gray intensity analysis of p-PI3K/PI3K, p-Akt/Akt, p-mTOR/mTOR, Beclin-1/ β -actin, LC3BII/LC3BI, p62/ β -actin, Bcl-2/Bax, and cleaved caspase-3/ β -actin. One-way ANOVA with Dunnett *post hoc* analysis was carried out to analyze the differences among groups using GraphPad Prism 5.0. $N = 5$ /group. ANOVA, analysis of variance; PI3K, phosphoinositide 3-kinase; SD, sleep deprivation; SLSP, stem-leaf saponins of *Panax notoginseng*. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.0001$ vs control group. # $P < 0.05$; ### $P < 0.01$, #### $P < 0.0001$ vs SD group.

particularly at higher doses (50 and 100 mg/kg), mitigated the expression of LC3B in the same regions. These results suggested that SLSP could hamper autophagosome formation in the hippocampus of SD mice.

2.4. SLSP regulated the expression of apoptotic and autophagic proteins in hippocampus of SD mice

To further confirm the effect of SLSP on autophagy, the expression of autophagy-related proteins and the activation of autophagy-related signaling proteins were examined by Western blot. As shown in Fig. 5, SD reduced the phosphorylation of PI3K, Akt, and mTOR, while increased the expression of Beclin-1, LC3B, and p62 in the hippocampus compared with the control ($P < 0.05$ or $P < 0.01$). Meanwhile, SD modulated the expression of apoptosis-related proteins, such as Bcl-2, Bax, and cleaved caspase-3. SLSP, especially used at higher dosages (50 and 100 mg/kg), could reverse the expression of autophagic and apoptotic proteins induced by SD.

These results indicated that SLSP could alleviate hippocampal neuronal autophagy and apoptosis of SD mice.

2.5. SLSP suppressed the autophagy and apoptosis of HT-22 cells induced by rapamycin

To examine if SLSP had direct protective effect on hippocampal neuronal cells, HT-22 cells, one of the hippocampal neuronal cell lines, were used. On rapamycin induction, there were much more acidic vesicular organelles (AVOs) appeared in bright red in the cytoplasm of HT-22 cells compared with the controls (Fig. 6A). 3-MA, the autophagy inhibitor, decreased the formation of cytoplasmic AVOs. Similarly, SLSP treatment at higher concentrations (25 and 50 $\mu\text{g/ml}$) could effectively reduce the density of cytoplasmic AVOs. To further confirm the antiautophagic effect of SLSP, the tandem fluorescent-tagged LC3 reporter, tf-LC3, was transiently transfected into HT-22 cells. As shown in Fig. 6B, rapamycin increased the numbers of autophagosomes indicated by GFP puncta and autolysosomes indicated by RFP puncta. SLSP treatment at

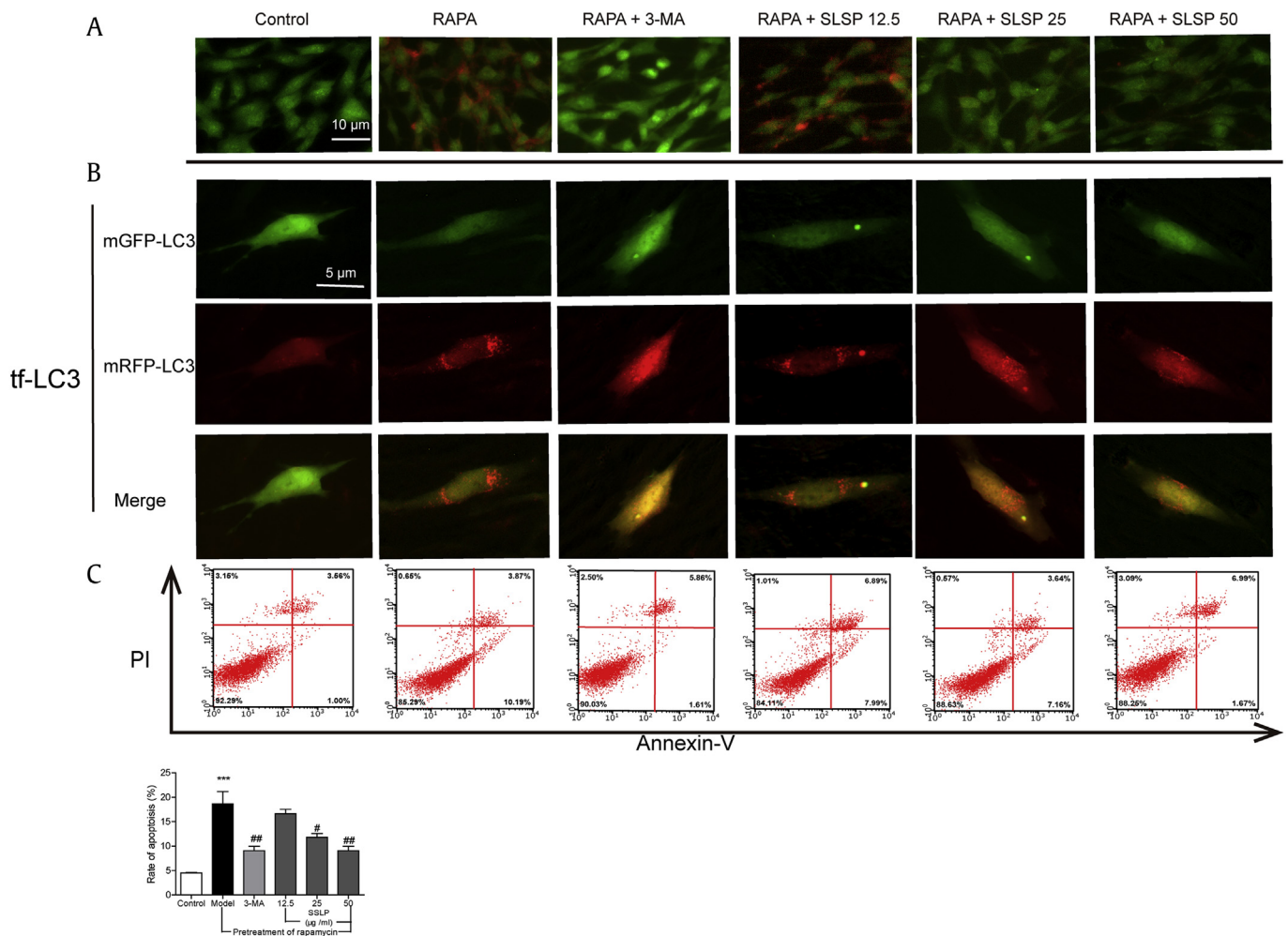


Fig. 6. The effect of SLSP on autophagy and apoptosis of HT-22 cells. (A) Acridine orange staining showed that SLSP could reduce acidic vesicular organelles appearing in bright red color in HT-22 cells induced by rapamycin. (B) The effect of SLSP on HT-22 transfected with tf-LC3. HT-22 cells were transfected with the tf-LC3 plasmid. After being pretreated with rapamycin (50 $\mu\text{g/ml}$) for 4 h, the cells were exposed to different concentrations (12.5, 25, 50 $\mu\text{g/ml}$) of SLSP for 48 h. (C) Annexin V/PI staining showed that SLSP could reduce the percentage of apoptotic HT-22 cells induced by rapamycin. The cells were incubated with different concentrations (12.5, 25, 50 $\mu\text{g/ml}$) of SLSP for 48 h after being pretreated with rapamycin (50 $\mu\text{g/ml}$) for 4 h and then subjected to annexin V/PI staining. (D) Comparison of the percentage of apoptotic HT-22 cells. The total percentage of cells in the right upper quadrant (late apoptotic cells) and lower quadrant (early apoptotic cells) of the respective flow cytometry chart was used for the statistical analysis. One-way ANOVA with Dunnett *post hoc* analysis was carried out to analyze the differences among groups using GraphPad Prism 5.0. ANOVA, analysis of variance; SD, sleep deprivation; SLSP, stem-leaf saponins of *Panax notoginseng*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ vs control group. ## $P < 0.01$; ### $P < 0.0001$ vs. SD group.

higher concentrations (25 and 50 $\mu\text{g/ml}$) reduced the number of green fluorescence punta and that of the red fluorescence punta, indicating that SLSP could inhibit the formation of autophagosome, thereby, reduce the formation of autolysosome.

To explore if SLSP had any effect on the apoptosis of hippocampal neuronal cells, HT-22 cells induced with rapamycin followed by SLSP and 3-MA treatment were stained with PI/annexinV and subjected to flow cytometry assay. As shown in Fig. 6C, rapamycin induction increased the percentage of apoptotic cells, which was significantly reduced by 3-MA treatment. SLSP treatment also prevented the increase of apoptotic cells induced by rapamycin. These results robustly implicated that SLSP could effectively suppress the autophagy and apoptosis of hippocampal neuronal cells.

2.6. SLSP modulated the expression of autophagic and apoptotic proteins in HT-22 cells induced by rapamycin

To further confirm the effect of SLSP on HT-22 cells induced by rapamycin, the proteins involved in the cellular autophagy and apoptosis were examined by Western blotting assay. As exposed in Fig. 7, compared with untreated cells, rapamycin-induced HT-22 cells showed reduced phosphorylation of PI3K, Akt, and mTOR while enhanced expression of Beclin-1, LC3B, and p62. Meanwhile,

rapamycin decreased the ratio of Bcl-2/Bax but increased the expression of cleaved caspase-3 within the cells. On the contrary, SLSP treatment could reverse the abnormal change of the molecules, implicating its antiautophagic and antiapoptotic effects on hippocampal neuronal cells.

To confirm that SLSP reversed rapamycin-induced autophagy and apoptosis through Akt/mTOR pathway, the Akt inhibitor VIII was used. As shown in Fig. 8, when Akt inhibitor VIII was added, SLSP could not abolish the autophagy and apoptosis anymore.

3. Discussions

Hippocampus is the critical brain region in charge of learning and memory. Accumulative evidence has demonstrated that SD induces spatial learning and memory impairment that resulted from the structural alteration of hippocampus, especially the sub-fields of hippocampus, such as CA3 and DG, which encode discrete, nonoverlapping memory representations [2,32–34]. Moreover, SD impairs neuronal plasticity at molecular and cellular levels at these hippocampal subregions [35]. As a positive control, modafinil can improve acquisition and reverse the memory impairment induced by SD [36–41], the process of which is probably through inducing hippocampal neuronal proliferation and differentiation [42].

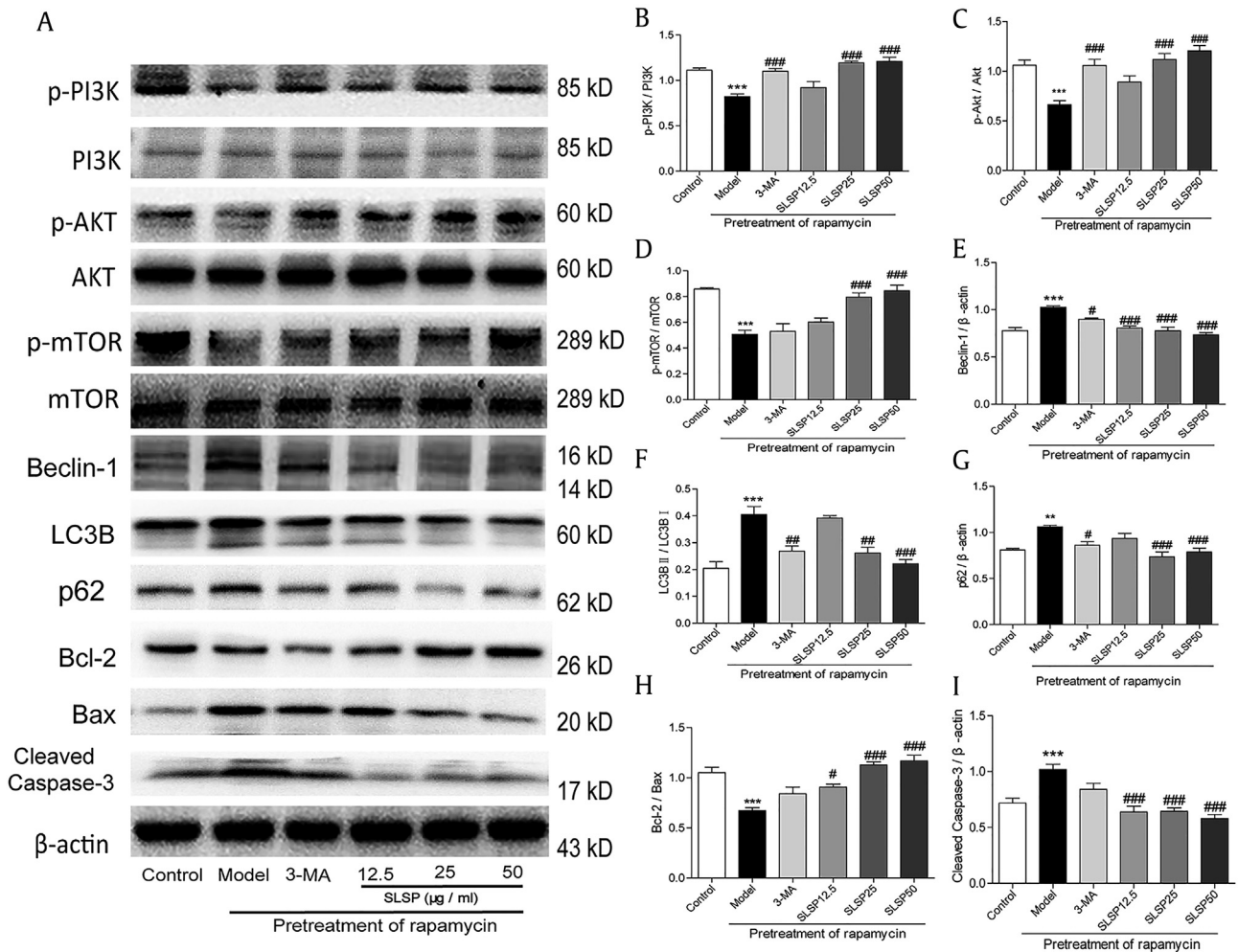


Fig. 7. The effect of SLSP on the proteins related to apoptosis and autophagy in HT-22 cells induced by rapamycin. (A) Western blotting bands of respective proteins in HT-22 cells. (B)–(I) Gray intensity analysis of p-PI3K/PI3K, p-Akt/Akt, p-mTOR/mTOR, Beclin-1/ β -actin, LC3BII/LC3BI, p62/ β -actin, Bcl-2/Bax, and cleaved caspase-3/ β -actin. One-way ANOVA with Dunnett *post hoc* analysis was carried out to analyze the differences among groups using GraphPad Prism 5.0. N = 5/group. ANOVA, analysis of variance; PI3K, phosphoinositide 3-kinase; SD, sleep deprivation; SLSP, stem–leaf saponins of *Panax notoginseng*. *P < 0.05; **P < 0.01, ***P < 0.0001 vs control group. #P < 0.05; ###P < 0.01, ####P < 0.0001 vs SD group.

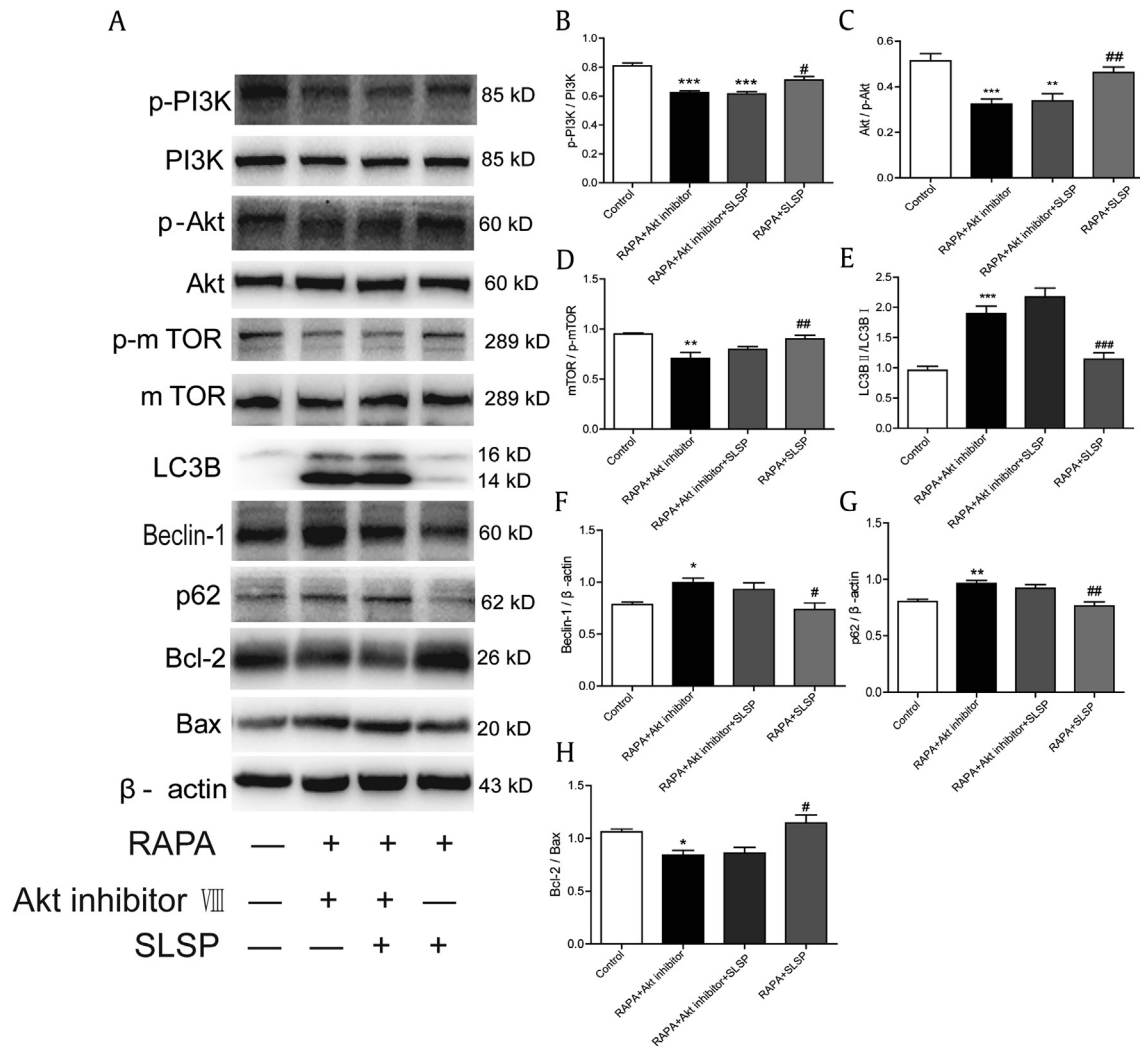


Fig. 8. The effect of SLSP on the proteins related to apoptosis and autophagy in HT-22 cells induced by rapamycin and Akt inhibitor. After being pretreated with rapamycin (50 µg/ml) for 4 h, the cells were exposed to SLSP (50 µg/ml) or SLSP (50 µg/ml) and Akt inhibitor (20 µmol/L) for 48 h. (A) Western blotting bands of respective proteins in HT-22 cells. (B)–(H) Gray intensity analysis of p-PI3K/PI3K, p-Akt/Akt, p-mTOR/mTOR, Beclin-1/β-actin, LC3BII/LC3BI, p62/β-actin, and Bcl-2/Bax. One-way ANOVA with Dunnett *post hoc* analysis was carried out to analyze the differences among groups using GraphPad Prism 5.0. N = 5/group. ANOVA, analysis of variance; PI3K, phosphoinositide 3-kinase; SD, sleep deprivation; SLSP, stem-leaf saponins of *Panax notoginseng*. *P < 0.05; **P < 0.01, ***P < 0.0001 vs control group. #P < 0.05; ##P < 0.01, ###P < 0.0001 vs SD group.

Consistent with the reports, in the present study, SD was found to impair spatial navigation of mice, accompanied with the shrunken morphology of neurons at CA3 and DG of hippocampus. Meanwhile, SD caused significant changes of autophagy and apoptosis related proteins at these subregions. In addition, SLSP treatment, particularly administered at higher dosages (50 and 100 mg/kg), could effectively rescue the abnormally altered symptoms induced by SD, from the behavior to molecular level, suggesting neuroprotective effect of SLSP on SD mice.

Autophagy is a lysosome-mediated degradation process in which dysfunctional organelles and macromolecules are sequestered into autophagic vesicles and delivered to lysosomes. Ultimately, the autophagic cargo molecules are degraded and recycled back to maintain cellular bioenergy homeostasis and support biosynthetic processes under metabolic stress conditions, including nutrient deprivation [43,44]. Sustaining the basal autophagy is beneficial for the neurons to eliminate dysfunctional proteins or organelles, whereas impaired autophagy or excessive autophagy may induce neurodegeneration as shown by extensive neuronal loss [44,45]. Many proteins, including LC3B, Beclin-1, and p62, actively participate in the autophagy process. LC3B is involved in

the formation of autophagosomes by mediating the wrapping of misfolded proteins and damaged organelles [46]. Beclin-1 primarily functions as a scaffolding protein to promote the formation of Beclin-1/Vps34 complex, thereby, accelerates the autophagy cascade [47]. p62 is a vital indicator of autophagic flux, which selectively incorporates into autophagosomes by directly binding to the LC3 on autophagic membranes for subsequent degradation in autolysosomes [48]. In the present study, an increased number of autophagosomes were found in the hippocampus of SD mice, which was accompanied with elevated expression of LC3B, Beclin-1, and p62, indicating excessive autophagy induced by SD. However, SLSP treatment could decrease the number of autophagosomes and the expression of LC3B, Beclin-1, and p62 within hippocampus of SD mice. Meanwhile, the *in vitro* experiments conducted on HT-22 cells confirmed that SLSP could counteract the formation of autophagosomes and suppress the elevation of LC3B, Beclin-1, and p62 induced by rapamycin. Both the *in vivo* and *in vitro* experiments implicated that SLSP could restrain the excessive autophagy within hippocampal neurons.

PI3K/Akt/mTOR signal transduction pathway is the canonical pathway involved in the regulation of autophagy [49]. Activation of

mTOR, a downstream target of PI3K and Akt, promotes cell growth, differentiation, and survival, whereas inhibits autophagy process [50]. In the present study, the PI3K/Akt/mTOR signaling was found to be suppressed in the hippocampus of SD mice, which was reversed by SLSP treatment. Meanwhile, SLSP abolished the inhibition of rapamycin on PI3K/Akt/mTOR signaling in HT-22 cells. 3-MA is an inhibitor of PI3K signaling pathway that blocks the formation of autophagolysosomes and degradation of proteins [51]. These results suggested that SLSP probably prevented the neuronal autophagy through activating PI3K/Akt/mTOR signaling pathway. Apoptosis is widely regarded as a major mechanism in the regulation of cell death initiated not only on cell damage or stress but also during normal development and morphogenesis [52]. Excessive apoptosis of certain postmitotic cells such as neurons contributes to neurodegeneration [52]. Both Bcl-2 and Bax belong to Bcl-2 family members but play opposite role, respectively, in intrinsic (mitochondrial) apoptotic pathway [53]. The balance of the two proteins determines the fate of cells toward survival or apoptosis [53,54]. Caspase-3 is the main executioner of caspase family members, which plays pivotal role in many cell apoptosis events [55]. In the present study, significant neuronal apoptosis was found within the hippocampus of SD mice shown by elevated cleaved caspase-3 but decreased Bcl-2/Bax ratio, which could be counteracted by SLSP treatment. On HT-22 cells induced by rapamycin, SLSP significantly inhibited cleaved caspase-3 and enhanced the ratio of Bcl-2/Bax. In addition, SLSP could decrease the percentage of apoptotic HT-22 cells on rapamycin stimulation. Therefore, both the *in vivo* and *in vitro* experiments indicated that SLSP could effectively alleviate the abnormal apoptosis of hippocampal neurons.

In summary, the present study disclosed that SLSP could alleviate cognitive impairment induced by SD, which was achieved probably through attenuating the abnormal autophagy and apoptosis of hippocampal neurons. The findings may contribute to the clinical application of SLSP in the therapy of neurological disorders associated with SD.

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Conflicts of interest

The authors declare no conflict of interest.

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