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ORIGINAL PAPER

Silibinin Alleviates the Learning and Memory Defects in Overtrained Rats Accompanying Reduced Neuronal Apoptosis and Senescence

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Abstract

Excessive physical exercise (overtraining; OT) increases oxidative stress and induces damage in multiple organs including the brain, especially the hippocampus that plays an important role in learning and memory. Silibinin, a natural flavonoid derived from milk thistle of *Silybum marianum*, has been reported to exert neuroprotective effect. In this study, rats were subjected to overtraining exercise, and the protective effects of silibinin were investigated in these models. Morris water maze and novel object recognition tests showed that silibinin significantly attenuated memory defects in overtrained rats. At the same time, the results of Nissl, TUNEL and SA-β-gal staining showed that silibinin reversed neuronal loss caused by apoptosis, and delayed cell senescence of the hippocampus in the overtrained rats, respectively. In addition, silibinin decreased malondialdehyde (MDA) levels which is associated with reactive oxygen species (ROS) generation. Silibinin prevented impairment of learning and memory caused by excessive physical exercise in rats, accompanied by reduced apoptosis and senescence in hippocampus cells.

Keywords Overtraining · Silibinin · Hippocampus · Apoptosis · Senescence

Introduction

For a long time, the benefits of sports have been deeply implanted in our perceptions, and in recent years more and more scientific researches have clearly confirmed the beneficial aspects of sports [1]. In addition to improving cardiopulmonary and cardiovascular function, exercise also effectively alleviates diseases closely related to body aging such as type 2 diabetes and Alzheimer's disease [2, 3]. However, excessive exercise with inadequate recovery period causes overtraining syndrome and results in damage in various body functions [4–6]. Overtraining syndrome disrupts the body's physiological systems, including the central nervous system (CNS) that causes depression, loss of concentration and impaired academic ability [7]. Highly strenuous exercise involved in marathon running induces impairment in explicit memory [8]. Cumulative evidence suggests that excess exercise causes impairment of cognitive behavior and damage on hippocampus [9].

The hippocampus, a structure essential for memory consolidation and emotion regulation, suffers from anatomical and functional changes during stress [10]. Actually, excess exercise induces a combination of physiological stresses, such as disturbances of metabolism, circulating hormones, body temperature, inflammatory state, and production of reactive oxygen and nitrogen species (ROS and RNS) [11–13]. Sliter and her coworkers reported a strong inflammatory phenotype caused by mitochondrial stress in the absence of parkin or PINK1 mice following exhaustive

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exercise [14]. Intriguingly, mitochondrial dysfunction in the mice brain caused by intense exercise was reported [15]. Both the increase in the production of ROS and the increase in the phenotype of inflammation promote the progression of aging [16, 17].

Cellular senescence signaling pathways involve p53–p21 and p16-retinoblastoma (Rb) pathways, activates the associated proteins in the pathways leading to the cell cycle arrest and finally developed senescence [18, 19]. The aging animals with the senescent cells accumulated usually suffer from the impairment of learning and memory, showing the deterioration on the cognitive function, while senolytics which induce apoptosis in senescent cells, but not non-senescent ones, can preserve cognitive function [20]. On the other hand, excessive apoptosis of nerve cells impaired learning and memory [21–23].

Silibinin has been accepted as a scavenger of free radicals [24], and widely used in the treatment of liver disease for many years [25]. It is reported that silibinin has estrogen-like effects, playing a protective role by regulating estrogen receptor activities in vitro and vivo [26, 27]. Silibinin inhibits the activation of aging-related proteins and excessive ROS production in D-galactose-induced senescent mice [16]. Furthermore, in mice with memory impairment induced by amyloid beta, silibinin exhibits the anti-inflammatory and anti-oxidative effects, as well as regulates the autophagic level [28, 29]. Treatment with silibinin ameliorates streptozotocin-induced brain energy metabolism and cholinergic function impairment [30]. In this study, we show that neuronal apoptosis and senescence in rats caused by overtraining can be protected by silibinin administration.

Materials and Methods

Animals

Sixty 8–10 weeks old male Sprague–Dawley rats weighing 240–270 g were obtained from Changsheng Biotechnology (Shenyang, Liaoning, China). They were housed at 22 °C with a 12/12 h light–dark cycle (8:00–20:00 lights on) and allowed access to food and water ad libitum. All experiments and procedures were carried out according to the Regulations of Experimental Animal Administration issued by State Committee of Science and Technology of China.

Reagents

Silibinin with 99% purity determined by HPLC was purchased from Jurong Best Medicine Material (Zhenjiang, Jiangsu, China). Resveratrol was purchased from Aladdin Industrial (L.A., CA, USA). Primary antibodies against p21, Rb, Bcl-2, Bax, caspase-3 and β-actin, as well as horseradish peroxidase-conjugated secondary antibodies, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Electrochemiluminescence (ECL) reagent was from Thermo Scientific (Rockford, IL, USA). Other chemicals and materials were commercially available.

Treatment

All rats were divided randomly into the following groups: sedentary group, overtraining (OT) group, OT plus three silibinin-treated groups (25, 50, and 100 mg/kg) and OT plus resveratrol-treated group (20 mg/kg). Resveratrol exhibits its neuroprotection and reduces exercise-induced oxidative damage and ameliorates fatigue in many studies [31–34]. In addition to neuroprotective and cardioprotective effects, resveratrol also has the influence on insulin, SIRT1, mitochondrial production or lipogenesis, being recommended as a supplement to sportsman [35]. Therefore, resveratrol is used as a positive control in this study. Overtraining or excessive physical exercise is done by swimming for 5 weeks (duration, load, and frequency of exercise increased gradually corresponding to overtraining protocol in Table 1) [36]. The rats were forced to perform overtraining with a sinker attached around the root of the tail. The swimming exercise was performed in stainless steel swimming pool with 150 cm in diameter 60 cm in depth filled with water at 31–33 °C. The rats were considered as exhausted when they became the state including all of the three following principles: (1) Uncoordinated movement in swimming. (2) Failure to rise to the surface of water or sinking over 10 s. (3) No escape reaction or turnover movement, when they were free from water. The swimming capacity was measured according to the swimming period until exhaustion. When the rats became exhausted, they were removed from the water and allowed to rest for 5 min. Then, the same training was continued until the total swimming time reached 60 min.

Silibinin or resveratrol suspended in 0.3% carboxymethylcellulose (CMC) solution were administrated by oral gavage daily prior to physical exercise. To detect learning ability and memory ability, Morris water maze and novel object-recognition tests were carried out, respectively (Fig. 1a). On the 44th day of the experiment, for western blotting and

Table 1 Training protocol

Week	Training time (min)	Sinker weight (g)	Number of daily sessions
1	60	0	1
2	60	8.5	1
3	60	15	1
4	60	15	2
5	60	15	3

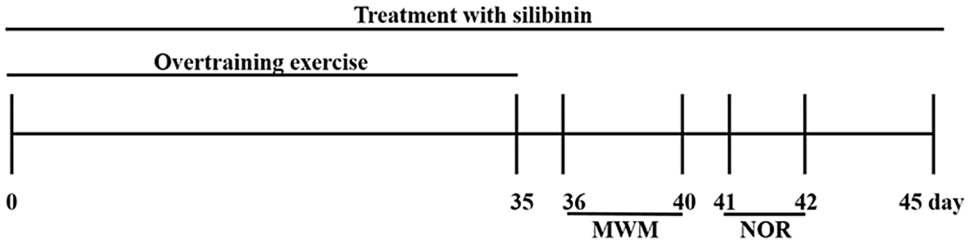

Fig. 1 Experimental protocol

oxidative stress determination, 24 rats (four in each group) were randomly selected and the hippocampus was immediately removed and placed in liquid nitrogen, followed by storage at − 80 °C. On the 45th day of the experiment, 24 rats (4 in each group) were intracardially perfused with 0.9% saline followed by pre-cooled 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and the brain was taken and post-fixed in 4% paraformaldehyde at 4 °C for 2 days. The brain was then divided into left and right hemispheres along the median sagittal plane for paraffin sections and frozen sections, respectively. All rats were weighed once a week.

Morris Water Maze Test

The rats were tested in the Morris water maze [37]. Briefly, a hidden platform 13 cm in diameter was placed 1.5 cm under the water surface in the target quadrant of a 150 cm diameter pool. The water was 20 ± 1 °C. Rats were trained twice daily for four consecutive days (day 36–39) with an inter-trial interval of 3 h and each trial lasted for 90 s. Rats were placed in the pool facing the wall in the other three quadrant, and escape latency was recorded with a video camera. On the fifth day (day 40) of the test, rats performed a probe test for 90 s without the platform. Swimming speed, platform-site crossings were recorded and analyzed by SLY-ETS type software (Beijing, China).

Novel Object Recognition Test

The novel object recognition test was performed on the 41st and 42nd days [28]. Briefly, two identical objects (A1, A2) were placed at a fixed distance in a 100 cm diameters open-field apparatus. Rats were placed into the field and allowed to explore 5 min, the exploring time for each object (tA1, tA2) was recorded. Then the rats were placed back into the same field after 24 h (object A2 was replaced with a novel object B), and the exploring time for each object (tA1, tB) was recorded for a 5 min session. Preferential index and discrimination index were calculated according to the following equations: preferential index = (tB)/(tA1 + tB); discrimination index = (tB − tA1)/(tA1 + tB).

Oxidative Stress Parameters in the Hippocampus

The hippocampus tissues were homogenized in ice-cold PBS. The supernatant was obtained by centrifugation of the homogenate at 2500×g at 4 °C for 20 min. Protein concentration was quantified using the BCA Protein Assay Kit (Beyotime, Jiangsu, China). The level of malondialdehyde (MDA), the activity of total superoxide dismutase (T-SOD) and the activity of catalase (CAT) were determined by using commercial reagent kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Western Blotting Analysis

The hippocampus samples were prepared for western blotting analysis, as described previously [37]. Tissues were homogenized in ice-cold whole cell RIRA lysis buffer (Beyotime, Jiangsu, China), and the protein concentrations were quantified using the BCA Protein Assay Kit. Equal amounts of protein (40–60 μg) samples were separated using 8–13% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. After incubation with primary antibodies against p21 (1:500), Rb (1:600), Bcl-2 (1:800), Bax (1:600) and β-actin (1:1500), the protein bands were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies, and then visualized with enhanced chemiluminescent ECL reagents.

The Staining of Senescence-Associated β-galactosidase (SA-β-gal)

After fixation in 4% paraformaldehyde at 4 °C for 2 days, the halves of brain were transferred to 20 and 30% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4 °C, respectively. After that, 30 μm thick frozen coronal section (three sections per rat) were cut and rehydrated three times, 5 min each, with PBS in a 24-well plate. Sections were then immersed in a fixation solution for 20 min and subsequently rinsed with PBS three times. Then 1 ml per well of working solution of β-galactosidase with X-Gal was placed and the plates were maintained at 37 °C overnight (senescence-associated β-galactosidase staining kit from Beyotime, Jiangsu, China). The sections were examined with an optical microscope. Photographs were analyzed by the software of Image-Pro

Plus 6.0. SA-β-gal positive ratio was expressed as the percent of SA-β-gal positive cells over the total cells.

Nissl Staining

Nissl staining was used for the detection of Nissl body in the cytoplasm of neurons. The staining method has been commonly used to identify basic neuronal structures in brains. Briefly, the other half of cerebral slices (2 mm) were obtained from bregma − 2.0 to − 6.0 mm, dehydrated in alcohol, permeabilized with xylene, embedded in paraffin. And 4 μm thick serially coronal sections (three sections per rat) were dewaxed and rehydrated by using decreasing gradient of ethanol, then were submerged in cresyl violet staining solution for 10 min. Nissl-positive cells in the hippocampus CA1 region were examined to assess neuronal loss. Representative photographs were captured and analyzed with the Image-Pro Plus 6.0 image analysis system.

Immunohistochemical Staining of Caspase 3

The 4 μm thick serially coronal paraffin sections were dewaxed and rehydrated by using decreasing gradient of ethanol. Sections were incubated overnight with anti-caspase 3 antibody (1:300; Santa Cruz, CA, USA), and then incubated for 1 h with biotinylated secondary antibody (Santa Cruz, CA, USA). The sections were developed with DAB staining (ZSGB-BIO, Beijing, China) and then counterstained with hematoxylin. The number of caspase 3 positive neurons was analyzed with Image-Pro Plus 6.0 analysis software.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining

The TUNEL staining was performed by using TUNEL Assay Kit (KeyGEN BioTECH, Jiangsu, China) according to the manufacturer's protocol. The 4 μm thick serially coronal paraffin sections were dewaxed with xylene, hydrated with a gradient ethanol, rinsed with PBS, and treated with proteinase K working solution. A total of 50 μl of the TUNEL reaction mixture was used for 60 min at 37 °C. Then the sections were incubated in Converter-POD solution and stained with DAB. After counterstained with hematoxylin, the sections were examined with an optical microscope. Apoptotic cell number in hippocampus CA1 area was assessed by counting the number of TUNEL-positive cells per unit area in three fields of each section (three sections per rat).

Statistical Analysis

Statistical analyses were determined by one- or two-way ANOVA followed by fisher's LSD multiple comparisons test using Statistics Package for Social Science software (version 13.0; SPSS, Chicago, IL, USA). Data are expressed as means ± S.E.M. The P values of < 0.05 were considered statistically significant. We also used Paired sample t-test to compare the exhaustion time of third week with the fifth week. Image-Pro Plus 6.0 and image J were used for image analysis.

Results

Silibinin did not Influence the Body Weight and Exercise Capacity of the OT Rats

From the second week onwards, the rats were subjected to weight-bearing swimming training with 8.5 g caudal dumbbell. With the exercise duration and workload gradually increased, the body weight of the OT rats was significantly reduced compared with the sedentary control group rats, and even negative growth occurred in the fourth and fifth week. Both silibinin and resveratrol did not affect the body weight of the exercised rats (Fig. 2a). Weight-bearing swimming

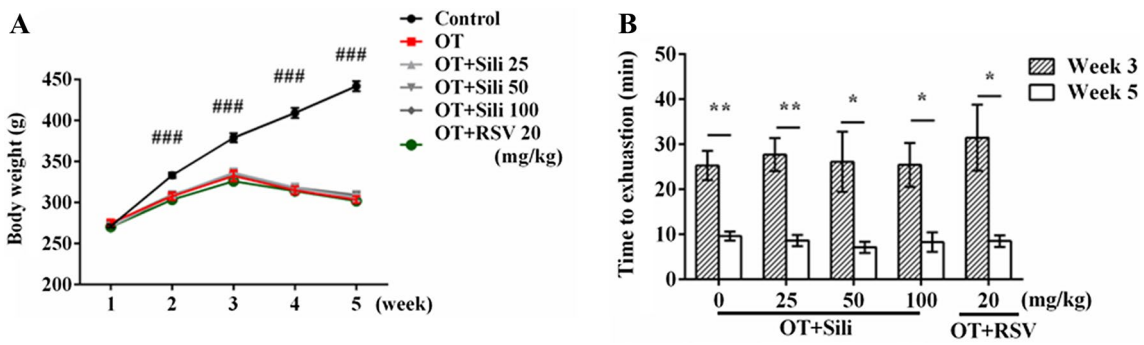

Fig. 2 Body weight and exercise performance of rats. **a** The effect of OT on body weight of rats. Results are expressed as means ± S.E.M. n = 6; $^{###}P < 0.001$ versus control. **b** The effect of OT on performance of rats. Time to exhaustion is significantly longer in the third week than in the fifth week. Data are expressed as means ± S.E.M. n = 6; * $P < 0.05$, ** $P < 0.01$

training with 15 g caudal dumbbell, started from the third week, and exhaustion occurred within 60 min. The exercise capacity of the rats was evaluated by swimming exhaustion time. Under the same conditions of the load, the exhaustion time of rats at the fifth week was significantly shortened compared with the third week, and administration of silibinin or resveratrol did not significantly increase the exhaustion time of the rats (Fig. 2b).

Silibinin Ameliorated the OT-Induced Learning and Memory Impairment in Morris Water Maze Test

During 4 days training period, there was a difference in the performance of six groups [F_{group} (5,120) = 3.831, $P < 0.001$; F_{day} (3,120) = 58.325, $P < 0.01$; $F_{group \times day}$ (15,120) = 0.332, $P = 0.991$, Fig. 3a]. From day 2 in the training test, OT rats took longer time to find the platform compared to sedentary control rats, and silibinin-treatment (100 mg/kg) decreased the escape latency ($P < 0.05$). The findings suggest that the learning ability was reduced by overtraining, but treatment with silibinin significantly prevents the defect. In the probe test, there was no difference in the swimming speed among the groups [$F(5,30) = 1.691$, $P = 0.167$, Fig. 3b]. The search accuracy of OT rats was obviously reduced [$F(5,30) = 2.402$, $P < 0.05$, post hoc, $P < 0.01$, Fig. 3c], suggesting an impairment of memory, but silibinin-treated (100 mg/kg) and resveratrol-treated rats showed better memory in their search accuracy as indicated by higher number of platform crossings compared to the OT rats ($P < 0.05$). Data indicated that silibinin and resveratrol improved the ability of spatial memory in OT rats.

Silibinin Prevents OT-Induced Impairment of Recognition Ability in Novel Object Recognition Test

The recognition and memory ability of the rats were investigated by novel object recognition test, and the preferential and discrimination indexes of the new objects were analyzed. Compared with the sedentary control group, the overtraining group showed a significant decrease in the preferential and discrimination indexes of new objects [$F(5,32) = 4.386$, $P < 0.01$, post hoc, $P < 0.001$; $F(5.32) = 4.386$, $P < 0.01$, post hoc, $P < 0.001$, Fig. 4a, b], while treatment with silibinin (50, 100 mg/kg) or resveratrol markedly sustained the preferential and discrimination indexes ($P < 0.01$; $P < 0.01$ or

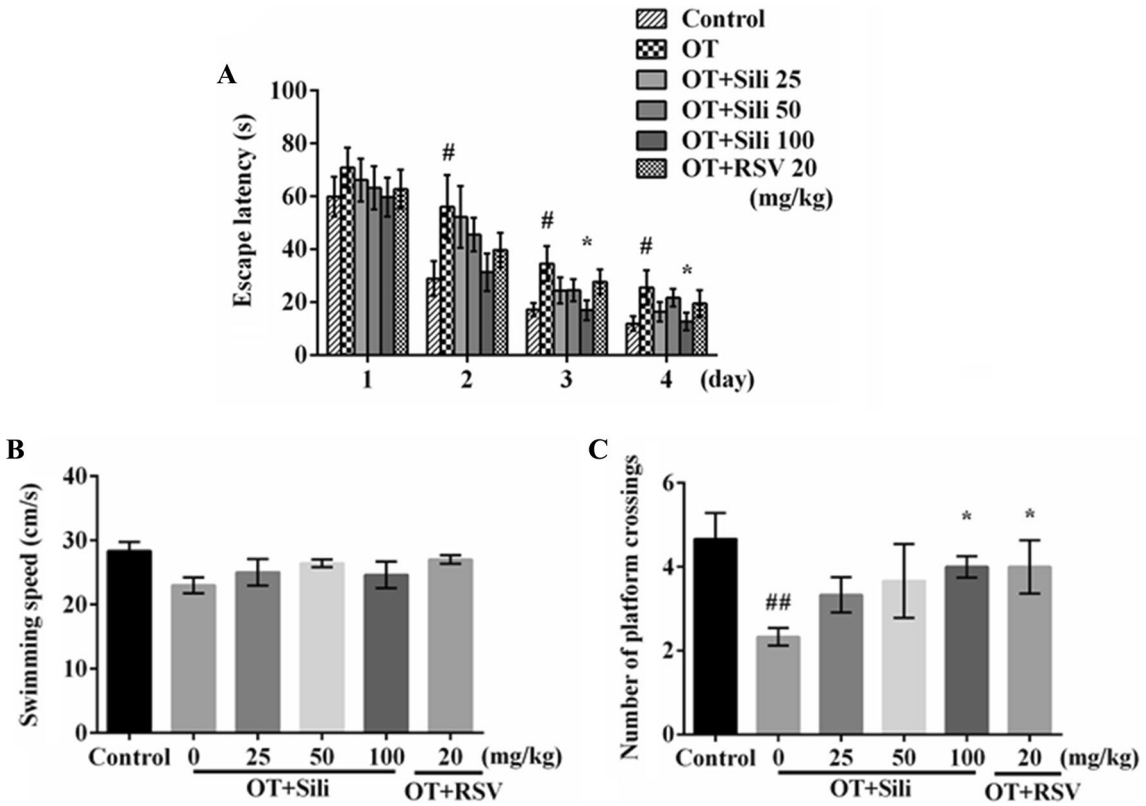

Fig. 3 Effects of silibinin on learning and memory deficits caused by OT in the Morris water maze test. **a** Changes in the latency to reach the platform during the training period. **b** Swimming speed among the groups. No significant difference among groups. **c** The number of platform crossings during the probe trail. Data are expressed as means ± S.E.M. n = 6; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Control rats; $^{*}P < 0.05$ versus OT rats

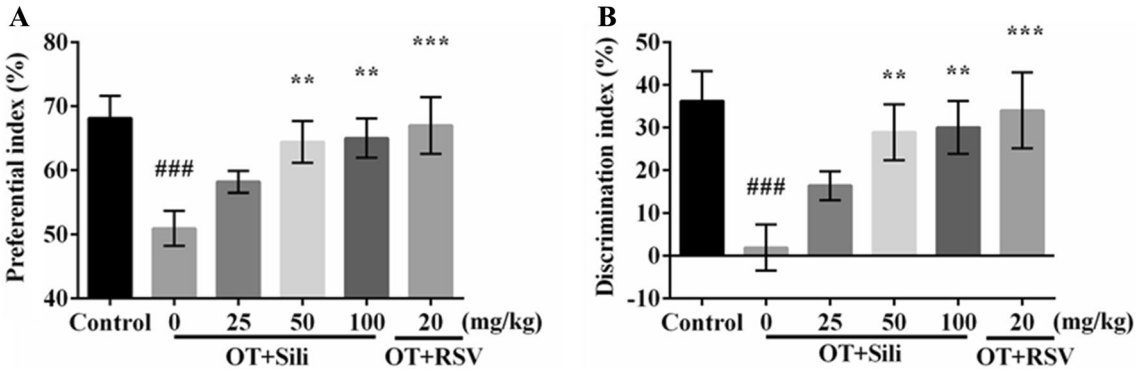

Fig. 4 Effects of silibinin on OT-induced recognition and memory impairment in novel object recognition test. **a** The preferential index for the novel object in test session. **b** The discrimination index for the novel object in test session. Data are expressed as means ± S.E.M. n = 6–7; $^{###}P < 0.001$ versus Control; $^{**}P < 0.01$, $^{***}P < 0.001$ versus OT rats

$P < 0.001$; Fig. 4a, b). The experimental results show that silibinin and resveratrol sustained the recognition and memory ability of OT rats.

Silibinin Repressed Neuronal Loss Caused by Apoptosis in Hippocampus of OT Rats

As shown in Fig. 5, OT resulted in an obvious neuronal loss and damage of neuron structure in the DG [$F(5,12) = 4.367$, $P < 0.05$, post hoc, $P < 0.01$, Fig. 5a], CA3 [$F(5,12) = 4.096$, $P < 0.05$, post hoc, $P < 0.01$, Fig. 5b] and CA1 [$F(5,12) = 14.991$, $P < 0.001$, post hoc, $P < 0.001$, Fig. 5c] region of the hippocampus, as reflected by density and morphology of the cells with Nissl bodies [38]. Consistent with the results of ethological analysis, the treatment with silibinin (100 mg/kg) reduced neuronal loss induced by OT ($P < 0.01$, $P < 0.05$, Fig. 5). We examined the expression of pro-apoptosis protein Bax and anti-apoptosis protein Bcl-2 in the hippocampus to investigate the neuronal cell death. Results show that the ratio of Bcl-2/Bax in OT group was remarkably lower than those of sedentary control group [$F(5,12) = 20.560$, $P < 0.001$, post hoc, $P < 0.001$, Fig. 6a], silibinin (100 mg/kg)- and resveratrol-treated groups ($P < 0.05$, Fig. 6a). These results indicate that silibinin or resveratrol helped sustain the level of Bcl-2, resulting in escape from apoptosis. The immuno-histochemical analyses further show that compared with the sedentary control group, the number of caspase-3 positive cells in hippocampal CA1 region of the OT group were significantly higher [$F(5,12) = 6.712$, $P < 0.01$, post hoc, $P < 0.001$, Fig. 6b]. The number of caspase-3 positive cells was reduced by administration of silibinin (100 mg/kg) or resveratrol ($P < 0.01$, $P < 0.05$, Fig. 6b). The TUNEL-positive apoptotic cells in hippocampus CA1 area of OT rats increased compared with the sedentary control rats [$F(5,12) = 6.617$, $P < 0.01$, post hoc, $P < 0.01$, Fig. 6c]. The TUNEL-positive apoptotic cells decreased in the tissues of the rats treated with silibinin (100 mg/kg) or resveratrol ($P < 0.01$, Fig. 6c).

Silibinin's Impact on the Levels of the Malondialdehyde (MDA), the Activity of Catalase (CAT) and Total Superoxide Dismutase (T-SOD) in the Hippocampus

Excessive and strenuous exercises provoke an increase in oxidative stress, which may lead to muscular fatigue and other organ injuries. The levels of MDA, the CAT and T-SOD activity reflect the degree of oxidative damage. In the present study, the MDA level in the hippocampus of OT rats increased compared with the sedentary control group rats [$F(5,14) = 2.992$, $P < 0.05$, post hoc, $P < 0.01$, Fig. 7a]. The level of MDA was lowered by the administration of silibinin or resveratrol ($P < 0.05$, Fig. 7a). Regarding antioxidant enzymes, silibinin-treated (100 mg/kg) group show higher CAT activity compared with the OT rats ($P < 0.05$, Fig. 7b). However, there was no significant difference in T-SOD activity among the groups [$F(5,14) = 1.082$, $P = 0.412$, Fig. 7c]. The experimental results show that silibinin possibly reduces the oxidative damage of rat hippocampus induced by OT through the promotion of CAT activity. Resveratrol shows similar trend, though statistically insignificant.

Silibinin Reduces the Senescent Cells in OT Rat Hippocampus

The process of senescence frequently results from radical-mediated oxidative damage, and therefore we turned to analyze the senescence level in hippocampus of OT rats. When the cells enter the senescence state, the activity of SA-β-gal is up-regulated, which catalyzes the substrate X-Gal into a dark blue product. The staining results show that compared with the sedentary control group, there were deeper blue products

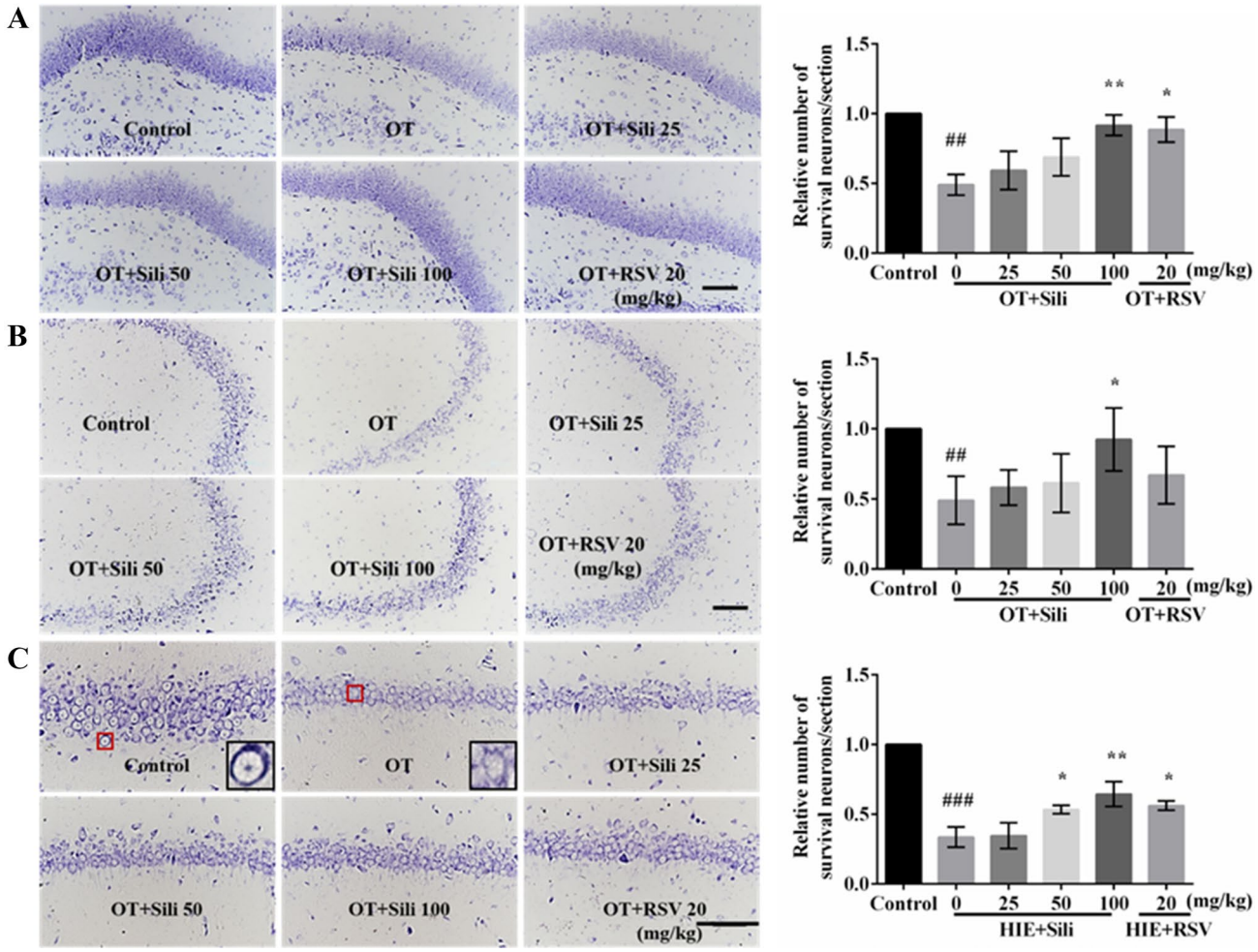

Fig. 5 Representative histological appearance of Nissl staining in the DG (**a**), CA3 (**b**) and CA1 (**c**) region of hippocampus. Silibinin treatment reduces OT-induced morphologic impairments and neuronal loss in the DG, CA3 and CA1 subfield, as reflected by the density of the cells with Nissl bodies. Surviving cells, characterized as normal morphology and clear nuclei and nucleoli were calculated statistically with Image-Pro Plus 6.0 analysis software. Data are expressed as mean ± S.E.M. n = 3; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. Control; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. OT rats. Scale bar = 100 μm

in the hippocampus, especially in the CA3 area of the OT rats [$F(5,12) = 5.527$, $P < 0.01$, post hoc, $P < 0.01$, Fig. 8a], indicating the existence of senescent cells. Apparent senescent cells were smaller in number by the administration of silibinin or resveratrol ($P < 0.05$, Fig. 8a). Moreover, western blotting analysis shows that compared with the sedentary control group, the expression levels of senescence-associated proteins p21 and Rb in the hippocampus of the OT group increased, but administration of silibinin and resveratrol down-regulated these proteins (Fig. 8b). These results show that silibinin or resveratrol inhibits OT-inducible senescence of rat hippocampal cells.

Discussion

The potential benefits of regular exercise training have been recognized for centuries and have been more definitively characterized in recent years [39]. However, it was reported that excessive exercise induced injuries in multiple organs, including lymphocyte system, kidney, and heart [6, 40, 41]. However, the toxic effect of excessive exercise on the central nervous system has not drawn widespread attention.

This study shows that there are significant weight loss and reduction of exercise performance in OT rats. The

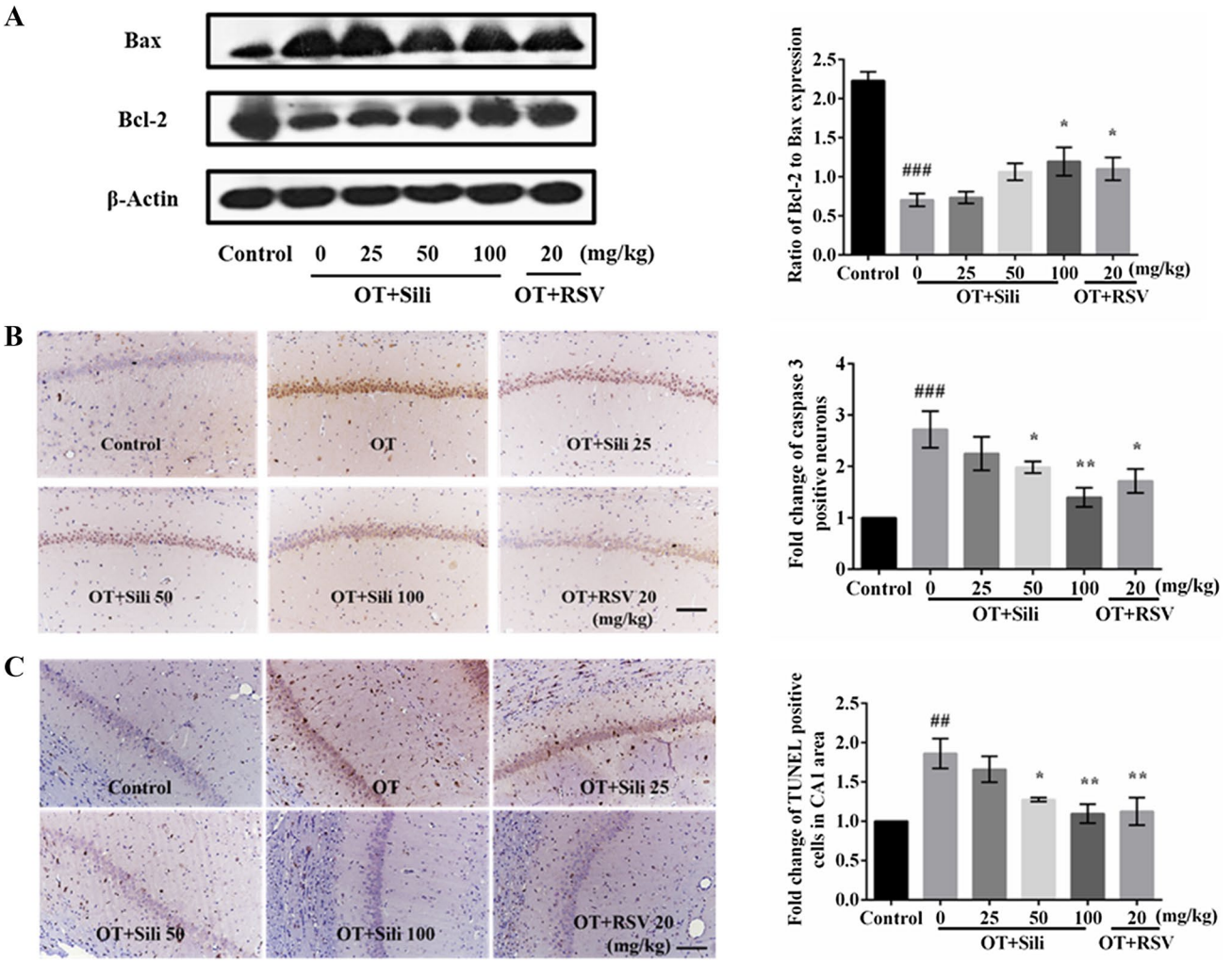

Fig. 6 Silibinin reduces OT-induced excessive apoptosis in hippocampus in rats. **a** Silibinin protects OT-induced decrease in Bcl-2/Bax ratio in the hippocampus. Quantified ratio of Bcl-2/Bax by Image J. **b** Silibinin reduced OT-induced caspase 3 expression in the CA1 regions with immuno-histochemical staining. **c** TUNEL staining showed that silibinin reduced OT-induced cells apoptosis in hippocampus CA1 areas, and the brown nuclei represented TUNEL-positive cells. The images were analyzed using Image-Pro Plus 6.0 analysis software. Data are expressed as means ± S.E.M. n = 3; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Control; $^{*}P < 0.05$, $^{**}P < 0.01$ versus OT rats. Scale bar = 100 μm

decrease in body weight is possibly related to hypercatabolism under persistent workloading [7]. A decline in exercise performance is due to a persistent combination of excessive overload plus inadequate recovery [42]. The European College of Sport Science and the American College of Sports Medicine jointly report overtraining syndrome as a continuous excessive overload plus inadequate recovery that reduces performance and various physiological functions [4]. Accordingly, it is most likely to conclude that OT rats experienced the overtraining syndrome. Training could have positive or negative effects on oxidative stress depending on training load, training specificity and the basal level of training [43, 44]. Excessive exercise increased oxidative stress and caused disruptions of the homeostasis. Brain is sensitive to oxidative damage because of the relatively low levels of both enzymatic and nonenzymatic antioxidant systems against a huge amount of free radical production due to the high oxygen consumption [45, 46]. Overall, regular moderate aerobic exercise appears to promote antioxidant capacity on brain. In contrast, anaerobic or high-intensity exercise, aerobic-exhausted exercise, or the combination of both types of training could deteriorate the antioxidant response [47]. In addition, it was reported that the regional hippocampal tissue oxygen pressure decreased to about 70% of sedentary level after the highly intensive swimming exercise [48]. Neurons are very sensitive to lack of oxygen and have been known to take damage by ischemia. According to ethological analysis, we found significant impairment of memory and deterioration on the cognitive function in OT

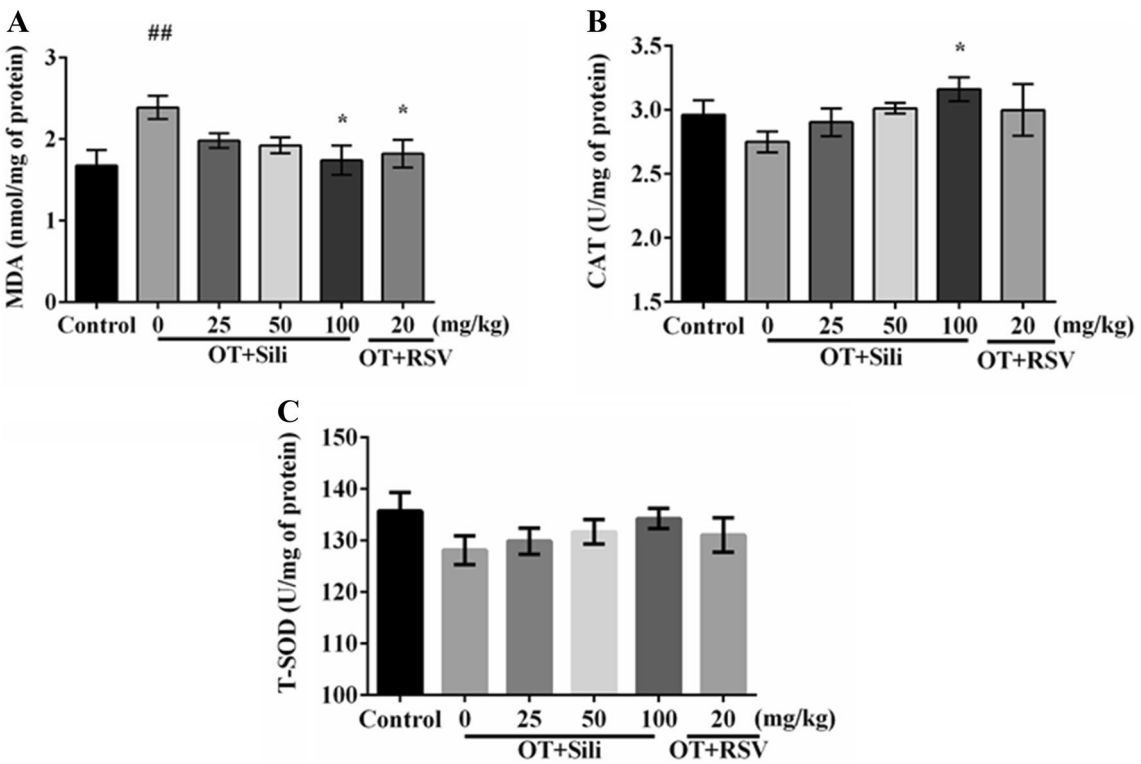

Fig. 7 Silibinin ameliorates oxidative stress caused by OT. **a** Silibinin-treatment significantly decreases MDA level in the hippocampus of OT-induced rats. **b** Silibinin-treatment promotes CAT activity in the hippocampus. **c** No significant differences in T-SOD activity among the groups. Data are expressed as means ± S.E.M. n = 3–4; $^{\#\#}P < 0.01$ versus Control; $^{*}P < 0.05$ versus OT rats

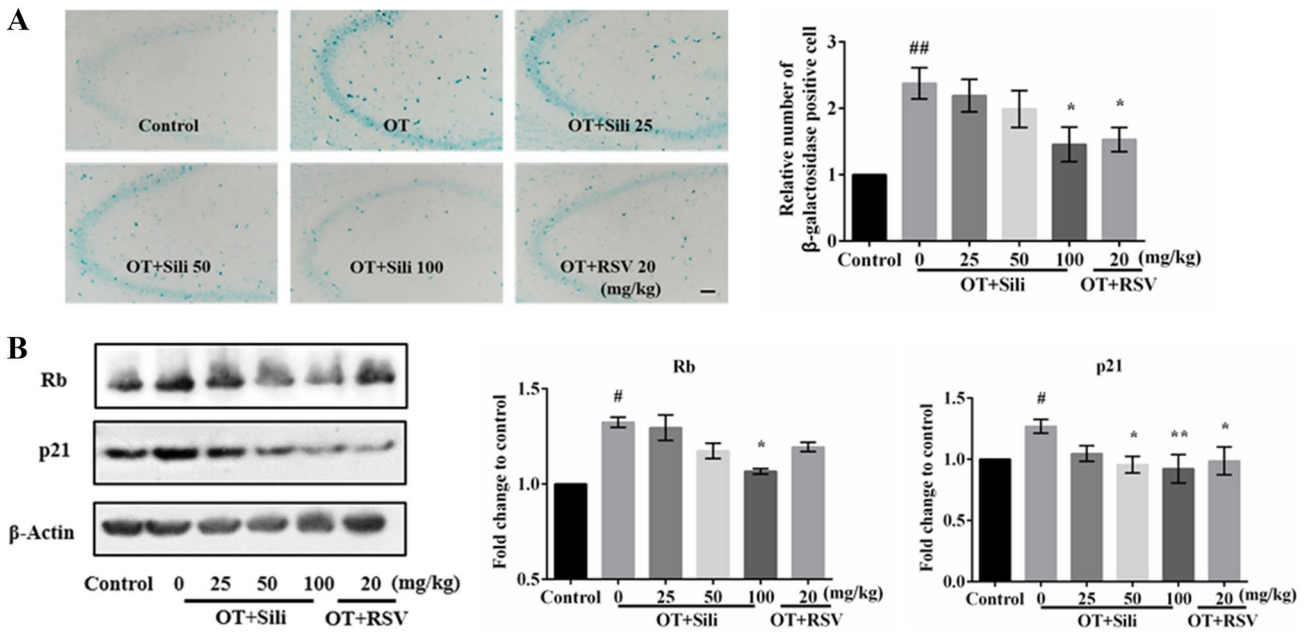

Fig. 8 Silibinin reduces OT-inducible senescent cells in rat hippocampus. **a** Effects of silibinin on SA-β-gal activity in the hippocampal CA3 region. The blue marker represents β-galactosidase. **b** Silibinin down-regulates the expression levels of Rb, p21 in the hippocampus. Quantification of Rb, p21 protein expressions was analyzed by Image J. Data are expressed as means ± S.E.M. n = 3; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ versus Control; $^{*}P < 0.05$ versus OT rats. Scale bar = 500 μm (Color figure online)

rats. Moreover, Nissl staining indicates that typical neuropathological changes are discernible in the hippocampus of OT rats, particularly in the CA1 region, such as cellular vague outline and confused boundary. Bcl-2 and Bax are functionally counterpart molecules in the Bcl-2 family. Bcl-2 is an important anti-apoptotic molecule and Bax is a critical pro-apoptotic one in the Bcl-2 family. A low ratio between Bcl-2 and Bax indicates the initiation and development of apoptosis [49], with a high caspase 3 expression, an indicator of apoptosis. In our study, together with the results of immunohistochemical and TUNEL staining, a greater percentage of neuronal apoptosis is identified in OT rats, which may result from the mitochondrial DNA impairment [50].

Increase in MDA levels in the hippocampus of OT rats when compared to the sedentary control group can be partially ascribed to lowered level of the antioxidant system consisting of antioxidant enzymes and non-enzymatic antioxidants. As for antioxidant enzymes, SOD catalyzes $O_2^{\cdot-}$ to H_2O_2, which is removed by other enzymes such as CAT and GPX [51]. Silibinin can ameliorate oxidative stress by increasing CAT activity. Intense physical training not only increases oxidative stress, but also activates the inflammatory response in rats [9]. Furthermore, excessive physical training causes mitochondrial dysfunction in the brain cortex of mice, followed by low cortical BDNF levels [15]. Oxidative stress, inflammation and nutritional or growth factor deficiency are closely related to aging [17, 52–54]. Senescent cells at the molecular level show increased expression of p53, p21, p16 and Rb [19, 55]. SA-β-gal is one of the most commonly used markers for cell-aging. Elevated expression levels of p21 and Rb in accordance with increased activity of SA-β-gal in the hippocampus of OT rats can be rationally accounted for by the concept that excessive exercise may accelerate aging process.

Although administration of silibinin or resveratrol does not increase the body mass and exercise capacity of OT rats, it can't be ruled out that silibinin also acts indirectly *in* preventing brain damage. This is one of the limitations of our study. On the other hand, it is reported that both silibinin and resveratrol cross the blood–brain barrier and exert neuroprotective in vivo [26, 56]. Resveratrol has anti-oxidant, anti-inflammatory and life longevity-extensive effects through activating sirtuin 1, AMP-activated protein kinase and nuclear factor erythroid 2-related factor 2 [57]. Furthermore, resveratrol improves brain mitochondrial function, which is weakened in Alzheimer's or other neurodegenerative diseases [34, 58, 59]. Silibinin, similar to resveratrol, shows neuroprotective effects in various degenerative nerve disease models [60–62]. Silibinin elevates the expressions of the BDNF and TrkB in the LPS-injected rats, and protects the rats from neuroinflammation and cognitive defects [37]. It is also reported that silibinin ameliorates memory impairment by regulating estrogen receptors in $A\beta_{1\text{-}42}$-injected rats [26]. Furthermore, activation of AMP-activated protein kinase signaling, improvement in brain energy and cholinergic function are involved of silibinin-mediated neuroprotection [30, 63].

However, further studies are needed in the future to define the pathway of OT-induced senescence and apoptosis as well as the protective mechanism of silibinin.

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