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The Anti-Liver Aging Effect of Salidroside Against Natural Aging C57 Mice by Regulating the Expression of Telomerase Reverse Transcriptase

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Abstract **Objective** To investigate the anti-liver-aging effect and mechanism of salidroside (Sal) against natural aging C57 mice by regulating the expression of telomerase reverse transcriptase (TERT). **Methods** A total of 50 C57 mice were randomly selected from 10 mice reared to 3 months old as the young control group, the other 40 mice were fed to 15 months of age as early aging mice, and were randomly subdivided into aging group, positive drug cycloastragenol (CAG) group, salidroside low-dose (Sal-L) and salidroside high-dose (Sal-H) group consisting of 10 mice each group respectively. The mice in young control group and aging group were given the same amount of normal saline 10 mL/kg intragastric administration, while the mice in positive drug group was given 20 mg/kg of CAG intragastric administration, and the mice in Sal-Land Sal-H groups were given 25 mg/kg and 100 mg/kg of salidroside respectively, once a day for 60 days. After the treatment, the aging degree of mice was scored. Colorimetric method was used to detect the activity of monoamine oxidase (MAO) in serum and the reactive oxygen species (ROS) content in serum. was detected by fluorescent probe method. The fibrosis of mouse liver tissue was observed by Masson staining and lipofuscin deposition was observed by aldehyde fuchsin staining. The protein expression of TERT was detected by Western Blot. **Results** Salidroside can reduce the aging score of aging mice, serum monoamine oxidase activity and the serum level of reactive oxygen species. The pathological staining indicated that salidroside could improve the aging degree of liver fibrosis and fat brown pigment deposition in mice.Western Blot results showed that the expression of TERT in liver tissue of aging mice increased after treatment with salidroside. **Conclusion** It is suggested that salidroside might delay the aging of mouse hepatocytes by up regulating the expression of TERT protein in the liver tissues of aging mice and inhibiting oxidative stress.

Keywords: *alidroside, senescence, hepatocyte, oxidative stress, telomerase reverse transcriptase*

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1. Introduction

Aging or Senescence refers to the gradual loss of molecular fidelity after reaching sexual maturity, resulting in loss of function and eventually leading to disease and death [1]. Telomere attrition, mitochondrial dysfunction,unstable genome and loss of protein homeostasis are the main hallmarks of aging, among which telomere attrition is an important factor affecting aging [2]. Telomeres are highly regulated dynamic complexes located at the ends of chromosomes and are synthesized by telomerase. Telomerase can use its own RNA as a template to synthesize telomeric repeat sequences to maintain the length of telomeres and protect the stability of chromosome ends, thereby avoiding premature cellular senescence and accelerating the process of age-related diseases [3]. Telomerase consists of the catalytic subunit telomerase reverse transcriptase (TERT) and the non-coding human telomerase RNA component (TERC). TERT and TERC are the catalytic core of telomerase. The key regulation of telomerase expression is the transcription of TERT gene. TERT also determines which cell types can express telomerase [4]. The transcriptional regulation of TERT is mainly dependent on the binding of Transcription factor (TF) to the promoter [5]. Modern pharmacological studies have shown that salidroside, an extract of *rhodiola rosea*, has anti-aging and antioxidant effects [6]. Mao et al. [7] found that salidroside can reduce the level of intracellular reactive oxygen species (ROS) in H2O2-induced cellular

senescence in a time-dependent manner, and alleviate its oxidative damage to functional molecules such as DNA and lipids in senescent cells. Zhu et al. [8] showed that after salidroside intervention, the activity of antioxidant enzymes increased, the level of oxidative damage related products decreased, and the expression of TERT protein in hippocampal tissue of aging mice increased. This study aims to establish a natural aging mouse model and investigate the anti-aging mechanism of salidroside from the aspects of oxidative stress, pathology and TERT protein expression.

2. Materials and Methods

2.1. Source of Materials

Fifty SPF male C57BL/6 mice, aged 2 months, weighing 22-24g, purchased from Beijing Weitong Lihua Laboratory Animal Technology Co. Ltd. (SCXK2016-0006). Salidroside (Sal), Shanghai Aladdin Reagent Co. Ltd. (S101157, purity ≥98%). Cycloastragenol (CAG), Shanghai Macklin Biochemical Technology Co. Ltd. (C860632, purity ≥98%). Masson staining kit (G1340), Beijing Solarbio Technology Co. Ltd. Aldehyde fuchsin staining kit (DJ0033), Beijing Leagene Biotechnology Co. Ltd. Monoamine oxidase (MAO) kit (TE0253), Beijing Leagene Biotechnology Co. Ltd. Reactive oxygen species (ROS) kit (BB475015), Yantai Bestbio Biotechnology Co. Ltd. TERT rabbit monoclonal antibody (DF7129) and β-actin rabbit polyclonal antibody (AF7018), Affinity Biosciences, USA.

2.2. Animal Grouping and Treatment

This experiment was approved by the Ethics Committee of Qingdao University Medical College (QYFY WZLL 27834). The local legislation regarding the ethics of animal experimentation and the guidelines for the care and use of laboratory animals, were followed in all animal procedures.

Fifty C57 mice were fed in SPF animal room with free diet. After feeding for 3 months, 10 mice were randomly selected as the young control group, and the other 40 mice were fed for 15 months. The mice were randomly divided into aging group, positive drug cycloastragalool (CAG) group, salidroside low-dose (Sal-L) group, salidroside high-dose (Sal-H) group. There were 10 rats in each group. The young control and aging groups were treated with normal saline 10 mL/kg by gavage, the positive drug group was treated with CAG 20 mg/kg by gavage, and the Sal-L and Sal-H groups were treated with Sal 25 mg/kg and 100 mg/kg by gavage, respectively, once a day for 60 days. The animal ethics was strictly followed during the experiment. All animals survived without death or loss.

2.3. Aging Degree Score

The aging degree scoring standard formulated by Tosuo Takeda and Masanori Hosokawa of Kyoto University [9] was used to observe 11 indexes such as behavior, skin and hair, eyes and spine of mice. Each index was divided into 3 to 5 grades and scored 1 to 5 points. The higher the score, the higher the aging degree.

2.4. Detection of Oxidative Stress Indicators

At the end of treatment, 5 mice were randomly selected from each group, and the mice were anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg), and 1.5 mL blood was collected from the heart (total protein was also extracted from liver tissue for Western blot, as described in later 1.6), after centrifugation at 4000 rpm for 5 min, the supernatant was taken to an EP tube and stored at 4 oC until use.

2.4.1. The activity of MAO was detected by aldehyde phenylhydrazone colorimetry: According to the instructions of MAO detection kit, a blank tube, a control tube, a standard tube, and a test tube were set up. The serum samples and solutions to be tested were added in turn, mixed, zeroed by distilled water, and the absorbance was measured at 470 nm by a spectrophotometer. The unit of MAO activity corresponding to benzaldehyde contained in the series standard was used as the abscissa, and the difference of absorbance was used as the ordinate to draw the standard curve. The MAO activity of the tested sample was found on the standard curve by the difference of absorbance of the tested sample, which was expressed as U/L.

2.4.2. BBoxiProbe®O12 fluorescent probe was used to detect ROS levels: The serum to be tested was taken and the O12 probe was diluted 10-fold with pure water according to the number of samples using the serum reactive oxygen species detection kit. The mixture was thoroughly mixed, and 100 μL serum sample, 10 μL O12 probe were added to a 96-well plate, and the mixture was thoroughly mixed by blowing through a pipettor. The cells were incubated at 37°C in the dark for 30 min. The fluorescence intensity was detected by fluorescence photometer (excitation wavelength 488 nm, emission wavelength 520 nm) to indicate the level of ROS.

2.5. Histopathological Examination

At the end of treatment, 5 mice from each group were randomly selected and anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg), then fixed by cardiac perfusion with 50 mL normal saline and 50 mL 4% paraformaldehyde, and 8×8×5 mm of right lobe liver tissue was obtained. Routine tissues were dehydrated, transparent, wax-soaked, embedded, sectioned (5 μm thick), pasted, and stored at room temperature until use.

2.5.1. Masson staining: The sections were deparaffinized and rehydrated, followed by hematoxylin staining for 5 min. The cells were washed thoroughly with distilled water and stained with Masson Ponceau acid fuchsine solution for 5 min. The cells were immersed in 2% glacial acetic acid for 1 min. 1% phosphomolybdic acid for 5 min. The slices were directly stained with aniline blue for 5 min, soaked with 0.2% glacial acetic acid for 1 min, dehydrated once with 95% ethanol, dehydrated three times with absolute ethanol, transparent with xylene, and sealed with neutral gum. Five non-overlapping high-power (×400) fields were randomly selected from each section under a microscope to observe the liver fibrosis of

mice. The degree of fibrosis was expressed as collagen volume fraction (CVF= collagen positive blue area/total tissue area).

2.5.2 Aldehyde fuchsin staining: Sections were routinely deparaffinized and rehydrated, treated with acidic oxidizing solution for 5 min, washed with distilled water, bleached with bleach solution for 2 min, and washed with distilled water. After washing with 70% ethanol, the mixture was added into aldehyde fuchsin staining solution, covered and stained, and washed three times with 70% ethanol and distilled water. They were routinely dehydrated, transparent, and sealed. Five non-overlapping high-power (×400) fields were randomly selected from each section under a microscope to observe lipofuscin deposition in mouse liver tissue. The degree of lipofuscin deposition was expressed as collagen volume fraction (CVF= dark purple area of elastic fibers/total tissue area).

2.6. Western Blot Was Used to Detect Protein Expression In Liver Tissue of Mice

After the end of drug administration, blood samples were collected from 5 mice in each group as described above, and livers were quickly collected by cardiac perfusion with normal saline and placed on ice, washed with PBS pre-cooled at 4°C, and the liver tissues were separated, weighed, cut into pieces, and placed in EP tubes. The mixture of RIPA lysate and protease inhibitor PMSF was added into the tube to lysate liver tissue, then centrifuged at 10,000 rpm for 5 min. The supernatant was removed, and the total protein concentration was determined by BCA kit. 10% separation glue and 5% concentration glue were prepared. After the glue was prepared, 20μg of protein sample was added to loading buffer for sample loading and heated at 95 oC for 6 min. After electrophoresis on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), the membrane was transferred to polyvinylidene difluoride (PVDF) membrane, and blocked with TBST buffer and skim milk powder (5% blocking solution). After blocking, the cells were incubated with the primary antibody (anti-TERT) and then incubated overnight in a refrigerator at 4 oC. The next day, the membranes were washed with TBST for 5min three times and incubated with HRP-labeled secondary antibody (1:10,000) for 1.5h at room temperature, after which the membranes were washed with TBST for 5min three times. Image J software was used to analyze the gray value of each band. The gray value of the same sample β-actin (42 kD) was used as an internal reference, and the ratio of the gray value of the target protein to the gray value of the internal reference protein β-actin was calculated.

2.7. Statistical Analysis

We first tested the homogeneity of variance of the samples, and all the data were tested to follow the normal distribution and homogeneity of variance. Therefore, one-way analysis of variance was used for data comparison between multiple groups, and LSD-*t* test was used for pairwise comparison. All data were statistically analyzed using Graphpad Prism 8.0 software, and values are presented as mean ± SD, $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of Salidroside on Aging Degree Scores In Mice

The aging score showed that the aging score of the aging group was significantly higher than that of the young control group ($P<0.01$). After treatment with Sal and CAG, the aging scores of CAG group, Sal-L group and Sal-H group were significantly lower than those of aging group ($F=30.98$, $P<0.01$). There was no significant difference among CAG group, Sal-L group and Sal-H group ($P>0.05$) There was no significant difference between Sal-L group and Sal-H group ($P> 0.05$)(Figure 1).

Group	Aging score	MAO activity (U/L)	ROS level (OD)
Young group	0.26±0.45	35.68±3.20	9.92±0.67
Aging group	9.26±3.05**	99.69±6.655**	28.89±2.36**
CAG group	4.39±1.30##	57.34±2.51##	15.14±1.61##
Sal-L group	5.26±1.38##	65.35±3.50##	16.53±2.16##
Sal-H group	3.39±0.73##	55.68±6.42##	14.31±2.22##
F value	30.98	71.36	52.11
P value	<0.0001	<0.0001	<0.0001

Compared with the young control group, **$P<0.01$; Compared to the aging group,##$P<0.01$

Figure 1. The aging score, MAO activity and ROS levels were compared among the groups (n=10)

3.2. Effect of Salidroside on Oxidative Stress Injury in Mouse Liver Tissue

The serum MAO activity of the aging group was significantly increased than that of young control group ($P<0.01$). After treatment with Sal and CAG, the serum MAO activities of CAG group, Sal-L group and Sal-H group were significantly lower than those of aging group ($P<0.01$). There was no significant difference among CAG group, Sal-L group and Sal-H group ($P>0.05$), but there was no significant difference between Sal-L and Sal-H groups ($P>0.05$)(Figure 1).

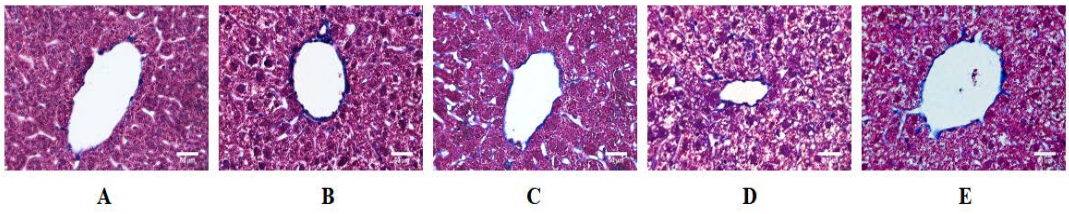
Compared with the young control group, the serum ROS level of the aging group was significantly increased ($P<.01$). After treatment with Sal and CAG, the serum ROS levels of CAG, Sal-L and Sal-H groups were significantly lower than those of aging group ($P<0.01$). There was no significant difference among CAG group, Sal-L group and Sal-H group ($P>0.05$), but there was no significant difference between Sal-L and Sal-H groups ($P>0.05$)(Figure 1).

3.3. Effect of Salidroside on Liver Fibrosis in Mice

Masson staining showed that the hepatocytes of the young control group were clearly visible, and the red fibers were arranged neatly, and there was no fibrous connective tissue hyperplasia. Compared with the young control group (0.15±0.02), the CVF of aging group (0.30±0.02) was significantly increased (F=17.07, P<0.01). After treatment with Sal and CAG, the CVF of CAG group, Sal-L group and Sal-H group decreased to (0.21±0.02), (0.24±0.03) and (0.19±0.03), and the differences were statistically significant (F=17.07, P <0.05, P<0.01). There was no significant difference among CAG group, Sal-L group and Sal-H group (P>0.05), but there was no significant difference between Sal-L and Sal-H groups (P>0.05)(Figure 2).

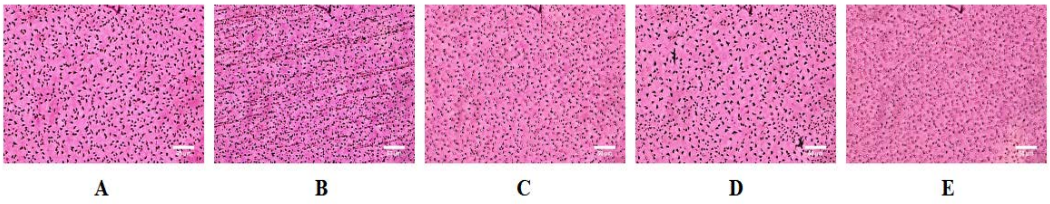
3.4. Effect of Salidroside on Lipofuscin Deposition in Liver Tissue of Mice

The results of aldehyde fuchsin staining showed that the liver cells of young control mice were clearly outlined and arranged neatly. Compared with the control group (0.12±0.01), the CVF (0.17±0.02) was significantly increased in the aged group (F=9.52, P<0.01). After treatment with Sal and CAG, the lipofuscin deposition in the liver tissue of CAG, Sal-L and Sal-H groups was improved compared with that of the aging group, the purple pigment granules in the cytoplasm were reduced, and the CVF was significantly reduced to (0.13±0.01), (0.15±0.03) and (0.12±0.01), respectively. The differences were significant (F=9.52, P<0.05, P<0.01). There was no significant difference among CAG group, Sal-L group and Sal-H group (P>0.05).There was no significant difference between Sal-L group and Sal-H group (P>0.05)(Figure 3).



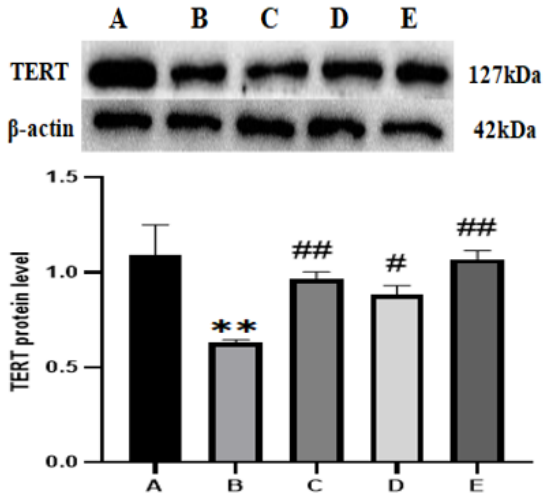
A: Young group, B: Aging group, C: CAG group, D: Sal-L group, E: Sal-H group, —: 50μm

Figure 2. Effect of salidroside on liver tissue fibrosis in mice (n=5), Masson×400



A: Young group, B: Aging group, C: CAG group, D: Sal-L group, E: Sal-H group, —: 50μm

Figure 3. Effect of salidroside on lipofuscin deposition in mouse liver tissue (n=5), with aldehyde fuchsin staining ×400



A：Young group, B：Aging group, C：CAG group, D：Sal-L group, E：Sal-H group

Compared with young controls, **P<0.01; Compared with the aging group, the #P<0.05, ##P< 0.01

Figure 4. Effect of salidroside on TERT protein expression in mouse liver tissues (n=5)

3.5. Salidroside Can Increase the Expression of TERT Protein in Liver Tissue of Aging Mice

Western Blot results showed that the expression of TERT protein in liver tissue of young control group was 1.09±0.16. Compared with the young group, the expression of TERT protein in the aged group was significantly decreased to 0.63±0.01 (P<0.01). After treatment with Sal and CAG, the expression of TERT protein in CAG group, Sal-L group and Sal-H group was significantly higher than that in aging group, and the differences were statistically significant (P<0.05). There was no significant difference among CAG group, Sal-L group and Sal-H group (P>0.05). There was no significant difference between Sal-L and Sal-H groups (P>0.05)(Figure 4).

4. Discussion

Aging is a physiological process in which the

physiological function of the body declines progressively and irreversibly. With the deepening of the aging process, a series of age-related diseases will occur, such as cardiovascular and cerebrovascular diseases, neurodegenerative diseases and cancer [10]. According to traditional Chinese medicine, liver is the first organ among the five-zang organs of the human body to begin aging, and liver aging marks the beginning of the human body to enter the aging stage [11]. Modern medicine believes that telomere attrition is an important factor affecting aging [12]. Salidroside (Sal) is an extract isolated from the rhizome of traditional Chinese medicine *Rhodiola*, which is the main active ingredient of *rhodiola* [13] and has anti-aging, anti-oxidation and anti-inflammatory effects. Cycloastragenol (CAG) is a guanidine glycan obtained by the hydrolysis of astragaloside, which has antioxidant and anti-inflammatory effects and is considered to be an effective anti-aging drug. Therefore, CAG was selected as the positive drug in this experiment [14].

The score of aging degree represents the degree of aging in mice, and the higher the score, the higher the degree of aging. The results of the present study showed that the aging scores of reactivity, fur gloss, degree of hair removal, and roughness of fur were significantly increased in the aging group compared with the young control mice. After treatment with salidroside and cycloastragenol, the aging scores of the CAG group and Sal group were significantly lower than those of the aging group. There was no significant difference between the Sal group and the CAG group, indicating that salidroside can effectively improve the degree of aging in aging mice.

With the aging of cells, mitochondrial dysfunction occurs and leads to a large amount of ROS production [15], and the level of adenosine triphosphate (ATP) decreases, which triggers the accumulation of oxidative stress damage and eventually induces apoptosis [16]. Previous studies have shown that the aging of liver tissue cells can lead to the accumulation of lipid in the tissue and the reduction of mitochondrial oxidative function, thereby generating a large amount of ROS [17]. The results of this experiment showed that the ROS level in the liver tissue of the aging group was significantly higher than that of the young control group, which was consistent with the results of previous experiments. After treatment with salidroside and cycloastragenol, the ROS levels of the mice were significantly lower than those of the aging group. There was no significant difference between the Sal group and the CAG group. Monoamine oxidase (MAO is an enzyme that can oxidize various monoamine neurotransmitters and hormones, and measurement of its activity can be used to evaluate the degree of oxidative stress injury in mouse liver tissue [18]. Han et al. [19] showed that the activity of MAO in the liver tissue of D-galactose-induced aging mice was very significant. The results of this experiment showed that the MAO activity in the liver tissue of the aging group was significantly higher than that of the young control group. After salidroside and cycloastragenol treatment, the MAO activity in the liver tissue of the mice was significantly lower than that of the aging group. There was no significant difference between the Sal-L and Sal-H groups, indicating that salidroside can effectively reduce the MAO activity and reduce oxidative stress damage in aging mice. The results of the above two oxidative stress indicators indicate that salidroside can effectively improve the antioxidant capacity of liver tissue in aging mice, reduce oxidative stress damage to liver tissue, and play an anti-liver aging role.

Li et al. [20] have shown that aging can cause dysfunction of various organs in the body, such as the liver, and increase the susceptibility of the liver to chronic inflammation and fibrosis. Among them, liver fibrosis causes serious damage and destruction to the liver and eventually leads to liver failure. Feng et al. [21] showed that one of the main characteristics of liver fibrosis is the excessive deposition of extracellular matrix (ECM) produced by continuous activation of hepatic stellate cell (HSC), and salidroside can inhibit HSC activation by inhibiting ECM production, thereby reducing the degree of liver fibrosis in mice. The results of this experiment showed that compared with the young control mice, the collagen fiber proliferation in the liver tissue of the aging mice was aggravated, the cell arrangement was disordered, and the liver fibrosis was more serious. After treatment with salidroside and cycloastragenol, the hepatocytes of mice were clearly visible and arranged in order, and the proliferation of collagen fibers was reduced. There was no significant difference in liver fibrosis between the Sal-L and Sal-H groups, indicating that salidroside can improve the liver fibrosis caused by aging in mice and reduce the degree of liver fibrosis in mice.

Lipofuscin is an endogenous fluorescent complex that accumulates over time and is one of the obvious characteristics of aging 22]. Studies have shown that aging leads to the accumulation of pigment granules (lipofuscin granules) in cells [23]. Aldehyde fuchsin staining was used to observe the lipofuscin deposition in the liver tissue of aging mice. Compared with the young control group, the liver cells in the aging group were atrophic, irregularly arranged, and a large number of lipofuscin particles were accumulated in the cytoplasm. After treatment with salidroside and cycloastragenol, the accumulation of lipofuscin particles in the liver tissue cells of the CAG group, Sal-H and Sal-L groups was significantly lower than that of the aging group, and the cell morphology was normal and arranged in order. There was no significant difference between the Sal-L and Sal-H groups. These results indicate that salidroside can improve lipofuscin deposition in liver tissue of aging mice and delay aging.

Telomere theory suggests that the main cause of aging is telomere shortening [24]. Carlos et al. [25] found that after telomere damage, replicative DNA polymerase could not complete the replication of eukaryotic eukaryotic DNA telomere region, so the length of telomere would be greatly shortened after several rounds of cell division, leading to genomic instability and then aging. Recent studies have shown that telomerase activation in adult or aging organisms has a positive effect on delaying aging [26]. Telomerase reverse transcriptase (TERT) is a catalytic subunit with reverse transcriptase activity, which can synthesize telomere TTAGGG repeats to maintain telomere length. Once telomeric repeats are added, the 3' end of the chromosome will be repositioned and telomerase can synthesize other repeats [27]. Two notable features of TERT are its ability to stabilize binding to telomerase RNA and to repeat reverse transcription RNA

template fragments [28]. In humans, telomerase holoenzyme expression and activity are regulated through a variety of pathways such as transcription, alternative splicing, phosphorylation, protein degradation and cellular localization. Some transcription factors can directly affect the promoter region of the TERT gene [29], leading to the up-regulation of the gene expression and telomerase activity [30]. The results of Western Blot showed that the expression of TERT protein in the liver tissue of the aged mice was significantly lower than that of the young mice. After treatment with salidroside and cycloastragenol, the expression of TERT protein in the CAG group, the Sal-L and Sal-H groups was significantly higher than that in the aging group, but there was no significant difference between the Sal-H and Sal-L groups. These results indicate that salidroside can up-regulate the expression of TERT protein in the liver tissue of aging mice.

In conclusion, salidroside can alleviate oxidative stress injury, improve the degree of liver fibrosis and lipofuscin deposition, and up-regulate the protein expression of TERT to exert its anti-aging effect in aging mice.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

S.N. and Z.L. conducted the study design and animal and histology experiments, and prepared the manuscript. S.N., H.S. and L.Z. designed the clinical experiment. T.L. and H.L. designed and supervised the research.

Data Availability Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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