Ginsenoside Rg1 Performs Anti-Aging Functions by Suppressing Mitochondrial Pathway-Mediated Apoptosis and Activating Sirtuin 3 (SIRT3)/Superoxide Dismutase 2 (SOD2) Pathway in Sca-1+ HSC/HPC Cells of an Aging Rat Model

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Background: Aging is characterized by progressive deterioration in metabolic and physiological process. The present research assessed the antagonistic effects and mechanisms of Ginsenoside Rg1 (Rg1) on aging of HSCs/HPCs.

Material/Methods: Fifty male Sprague-Dawley (SD) rats were treated and divided into the following groups: Control (n=10), Model (n=10, treated with D-galactose, as aging model), Rg1 Control (n=10), Rg1 treatment (n=10), and Rg1 prevention (n=10). An aging rat model was established by subcutaneous injection with D-gal. HSC/HPC cells were stained using SA-β-Gal staining. HSC/HPC cells were examined using flow cytometry assay. CFU-mix assay, with a few modifications, was performed. Cleaved caspase-3, B-cell lymphoma-2 (Bcl-2), and Bcl-2-associated X protein (Bax) were examined using qRT-PCR. Sirtuin 3 (SIRT3) and superoxide dismutase 2 (SOD2) expression was determined using Western blot assay and qRT-PCR.

Results: Rg1 (treatment and prevention group) significantly decreased SA-β-Gal-positive staining in Sca-1+ HSC/HPC cells compared to that of the D-gal model (p<0.05). Rg1 significantly enhanced formation capacity of CFU-Mix compared to the D-gal model (p<0.05) in Sca-1+ HSC/HPC cells. Rg1 significantly reduced G0/G1 phase of Sca-1+ HSC/HPC cells compared to that of the D-gal model (p<0.05). Rg1 significantly decreased cleaved caspase 3 and Bax expression, and increased Bcl-2 expression compared to the D-gal model (p<0.05). Rg1 treatment remarkably upregulated expressions of SIRT3 and SOD2 compared to that of the D-gal model group (p<0.05).

Conclusions: Rg1 conducted functions of anti-aging in Sca-1+ HSC/HPC cells in the D-gal-induced aging model by inhibiting mitochondrial pathway-mediated apoptosis and activating the SIRT3/SOD2 signaling pathway.

MeSH Keywords: Aging • Galactose • Ginsenosides • Hematopoietic Stem Cells • Sirtuin 3

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Background

Aging involves a series of biological processes and is characterized by progressive deterioration in metabolic functions and physiological roles [1,2]. These metabolic and physiological changes may cause higher mortality and morbidity. Aging of stem cells is considered to be caused by injury or damage of cells and tissues [3]. Previous studies [4,5] reported that reactive oxygen species (ROS) play the most critical role in progression of aging. Several studies [6,7] have reported that treatment resulting in increased anti-oxidant capacity can attenuate aging progression and the associated oxidative injuries.

Sirtuins are a highly conserved protein family that contains 7 NAD^+-dependent deacetylases [8]. These 7 deacetylases share the same catalytic domain, consisting of 275 amino acids [9]. Among these 7 NAD^+-dependent deacetylases, sirtuin 1 (SIRT1), sirtuin 6 (SIRT6), and sirtuin 7 (SIRT7) mainly localize to the nucleus, sirtuin 2 (SIRT2) is mainly distributed in cytoplasm, and sirtuin 3 (SIRT3), sirtuin 4 (SIRT4), and sirtuin 5 (SIRT5) are present in mitochondria [10]. Among SIRT3, SIRT4, and SIRT5 mitochondria-localized deacetylases, SIRT3 is the primary deacetylase that plays critical roles in cell apoptosis, energy metabolism, energy production, and some cell signaling pathways [11,12]. A few studies [13,14] also reported that SIRT3 has significant effects on ROS homeostasis of mitochondria, and directly regulates ROS production. A previous study [15] also showed that SIRT3 can change the levels of ROS through regulating the superoxide dismutase 2 (SOD2) acetylation levels. SOD2 deficiency is correlated with many human diseases and aging [16].

Rg1 is a ginsenoside that has been proven to be the most active ingredient of the herb Panax ginseng. Rg1 has many pharmacological functions, including anti-aging, anti-oxidation, and anti-injury effects [17]. A previous study by our team [18] showed that treatment with Rg1 can delay aging processes of HSCs or HPCs. However, the targeting regulatory molecules or signaling pathways involved in the aging processes of HSCs/HPCs cells have been unclear. Therefore, in the present study, we treated D-galactose-induced aging model rats with Rg1 to evaluate the antagonistic effects of Rg1 treatment on aging of HSCs/HPCs by measuring SA-β-Gal staining, evaluating apoptosis and CFU-mix of cells, and examining cleaved caspase-3, Bcl-2, and Bax expression. We also evaluated the regulatory function or mechanism of the SIRT3/SOD2 signaling pathway in Rg1-triggered aging antagonistic effects.

Material and Methods

Rats and trial grouping

A total of 50 male Sprague-Dawley (SD) rats (weight 210–250 g, age 3 months, certificate No. SCXK [Chongqing] 2007-0001) were provided by the Experimental Animal Center of the Chongqing Medical University. All rats were specific pathogen-free (SPF) animals. Rats were housed in an environment with light/dark cycle of 12 h/12 h at 25°C. All of experiments were approved by the Key Laboratory of Cell Biology in Yunnan, Kunming, China.

The 50 rats were divided into 5 groups with 10 rats each. In the Control group, rats were subcutaneously injected in the neck and back with saline (0.2 ml/rat/day) for 42 days. In the D-gal rat model group, rats were subcutaneously injected in the neck and back with D-gal (120 mg/kg/day) for 42 days. In the Rg1 control group, rats were intraperitoneally injected with saline (0.2 ml/rat/day) for 42 days, and then intraperitoneally injected with Rg1 (20 mg/kg/day) from day 8 to day 42. In the Rg1 treatment group, rats were subcutaneously injected in the neck and back with D-gal (120 mg/kg/d) for 42 days, and were intraperitoneally injected with Rg1 (20 mg/kg/day) from day 8 to day 42. In the Rg1 prevention group, rats were intraperitoneally injected with Rg1 (20 mg/kg/d) for 7 days, then subcutaneously injected in the neck and back with D-gal (120 mg/kg/d) for another 42 days, and then they were intraperitoneally injected with Rg1 (20 mg/kg/day) from day 8 to day 42. Rg1 with purity more than 95% was obtained from Jilin Hongjiu Bio. Tech. Co. (catalog no. 060427; Changchun, China). The D-gal was provided by Amresco Inc. (Solon, OH, USA).

Sample preparation

All rats were fed for 42 days and then anesthetized by intraperitoneal injections of pentobarbital. The Sca-1^+ HSC/HPC cells were harvested using the immunomagnetic separation method according to a previously described method [19]. The prepared samples were used for the following SA-β-Gal staining, flow cytometry assay, hematopoietic progenitor cell mixed colony (CFU-Mix) assay, and real-time PCR (RT-PCR) assay.

SA-β-Gal staining

HSC/HPC cells were collected in every group. To determine the effects of Rg1 treatment on cell aging, we used SA-β-Gal staining. The HSC/HPC cells were stained using SA-β-Gal staining regent (Cell Signaling Technology Inc., Beverly, MA, USA) based on protocols of the manufacturer. Cells were then centrifuged and cytospin slides were prepared. Then, slides were sheet-sealed using 70% glycerol (Sigma-Aldrich.). To observe and calculate the percentage of positive cells, 400 cells...
Table 1. Primers used in quantitative RT-PCR assay.

| Primers         | Forward (5’-3’)                   | Reverse (5’-3’)                   | Gene length (bp) |
|-----------------|-----------------------------------|-----------------------------------|------------------|
| Cleaved caspase 3 | GGACGCTGGGACCGTGGAAAAAA          | GCATGGCCTATACATGTGGCACG           | 159              |
| Bcl-2           | ATCGCCTCTGGATGCTGATGACTG          | AGAGACGAGGAGGAGAATCATAC           | 134              |
| Bax             | GACACCTGAGCTGAGCTGCTGAGCTGAGCTG  | GAGAGCACTGCTGGAGGAGAATCATAC       | 130              |
| SIRT3           | GCTTCTGGCGCGCTGATGACTG           | CACCCCTGCGCGCATCAC                 | 169              |
| SOD2            | GAGGGTGATGAGGGAGGAGAATCATAC      | GCAATGGCCTGGAGGAGAATCATAC         | 157              |
| β-actin         | GAGACCTTCAAACACCCCCAGG           | ATGCAGCAAGGATTCCG                 | 263              |

were selected randomly and counted in each slide. SA-β-Gal staining was conducted at least 6 times in each group.

Cell cycle analysis

To assess the effects of Rg1 on the cell cycle of HSC/HPC cells, cell cycle analysis was performed. The HSC/HPC cells were collected in all 5 groups, and washed using PBS. The HSC/HPC cells were incubated with 70% ice-cold ethanol overnight, and washed twice with PBS. Then, the HSC/HPC cells were treated with 1 mg/ml bovine pancreatic ribonuclease for 30 min at 37°C. Finally, the cells were stained using propidium iodide (PI, 50 μg/ml) for 30 min in the dark, and the stained cells were observed by flow cytometry. The cell cycle phases in the 5 groups were analyzed using multi-cycle software (PHENIX, Japan). Cell cycle assay was performed at least 6 times in each group.

CFU-mix assay

To explore the roles of Rg1 in growth of HSC/HPC cells, CFU-mix assay was performed using a previously described method [20], with a few modifications. The cells (1×10⁶ cells for every group) were incubated with DMEM (Gibco BRL. Co.) supplemented with 30% horse serum, 2 U/ml recombinant human erythropoietin (rhEPO, Kylin Kunpen Biol. Phar. Co., Shanghai, China), 10 ng/ml recombinant human granulocyte macrophage colony stimulating factor (rhGM-CSF, Kylin Kunpen Biol. Phar. Co.), 10 ng/ml intereleukin 3 (IL-3, Sigma-Aldrich), and 2.7% methylcellulose, with a final volume of 2 ml. The above components were thoroughly mixed, then were seeded into 96-well plates (0.2 ml/well) and cultured for 7 days at 37°C. The CFU-Mix formation capacity and multiple-differentiation capacity were evaluated according to the amounts of CFU-Mix and numbers of Sca-1+HSC/HPC. CFU-Mix assay was conducted at least 6 times in each group.

Quantitative RT-PCR assay

To assess the effects of Rg1 on expression of cell apoptosis-associated molecules, quantitative RT-PCR assay was conducted.

Total cellular RNAs were extracted using commercial TRIzol regents (Takara, Dalian, China). The Reverse Transcription kit (Takara) was then used to generate complementary DNAs (cDNAs) using on the protocols of manufacturer. The synthesized cDNAs were amplified as the templates using the Sybr green I (Takara) and ABI Fast real-time PCR system (ABI, Foster City, CA, USA). Then, the PCR assay was conducted with 35 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 30 s. Table 1 shows the specific primers amplifying targeting genes, including cleaved caspase 3, B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), SIRT3, SOD2, and β-actin. Images of amplified products were analyzed with Quantity One Imaging Software (Bio-Rad, CA, USA) and with the comparative threshold cycle (2⁻¹Ct) method [21]. The RT-PCR assay was conducted at least 6 times in each group.

Western blot assay

To confirmed the findings obtained from RT-PCR assay, Western blot assay was conducted to examine expressions of SIRT3 and SOD2. HSC/HPC cells were lysed with lysis buffer (Sigma-Aldrich), and the total proteins were extracted. The concentration of the proteins was examined using the BCA protein quantification kit (Beyotime Biotech, Shanghai, China). Equal amounts of protein were separated with 15% SDS-PAGE and electrotransferred onto PVDF membranes. Then, PVDF membranes were treated with 5% defatted milk for 4 h at 4°C. PVDF membranes were subsequently treated using rabbit anti-SIRT3 (cat. no: sc-99143), rabbit anti-SOD2 (cat. no. sc-30080), and rabbit anti-GAPDH (cat. no. sc-25778) for 2 h at 37°C. PVDF membranes were then treated using HRP-labeled anti-rabbit IgG (cat. no. sc-2030) for 60 min at 37°C. All of these primary and secondary antibodies were obtained from Santa Cruz Biotech. Eventually, Western blot images were visualized using the ECL Detection Kit (Pierce, Rockford, IL, USA). The Western blot assay was conducted at least 6 times in each group.

Statistical analysis

Data in this study were analyzed using SPSS software (version: 11.0, SPSS, Chicago, IL, USA) and are expressed as mean±SD.
Comparisons of variables (including cell cycle parameters, expression of cleaved caspase-3/Bcl-2/Bax, and SIRT3/SOD2 expression) among multiple groups were analyzed using ANOVA validated by Tukey’s post hoc test. The t test was utilized to compare differences of variables between 2 groups. A p < 0.05 value was considered as statistical significance.

**Results**

**Rg1 decreased SA-β-Gal-positive staining of Sca-1**

HSC/HPC cells

The results show that there were significantly more cells staining SA-β-Gal-positive in D-gal aging model rats than in the Control group (Table 2, p < 0.05). However, no obvious differences were found between the Control group and the Rg1 Control group (Table 2, p > 0.05). SA-β-Gal-positive staining cells in the Rg1 treatment group and Rg1 prevention group were significantly lower than in the D-gal aging model group (Table 2, both p < 0.05). Meanwhile, the SA-β-Gal-positive staining cells in the Rg1 prevention group was also remarkably lower compared to that of the Rg1 treatment group (Table 2, p < 0.05).

**Rg1 enhanced colony-formation capacity of CFU-Mix**

Compared to the Control group, the colony-formation capacity of CFU-Mix in the D-gal aging model was remarkably reduced (Table 2, p < 0.05); however, in the Rg1 Control group, it was remarkably enhanced (Table 2, p < 0.05). The colony-formation capacity of CFU-Mix in the Rg1 treatment group and Rg1 prevention group was also significantly increased compared to that of the Control group.

**Table 2. Effect of Rg1 on percentage of SA-β-gal staining positive cells and number of CFU-Mix forming of Sca-1+ HSC/HPC (±s, n=6).**

| Groups               | SA-β-Gal positive staining (%) | CFU-Mix amounts (1×10⁴ cells) |
|----------------------|-------------------------------|-------------------------------|
| Control              | 8.46±3.47                     | 25.76±3.44                    |
| D-gal aging model    | 51.2±4.82*                    | 8.21±3.36*                    |
| Rg1 control          | 5.74±3.66                     | 34.74±3.02*                   |
| Rg1 treatment        | 41.67±4.38*                   | 15.68±4.37*                   |
| Rg1 prevention       | 34.82±3.84*                   | 19.62±3.46*                   |

* p<0.05 compared to Control group; * p<0.05 compared to D-gal aging model group; # p<0.05 compared to Rg1 treatment group.

Comparisons of variables (including cell cycle parameters, expression of cleaved caspase-3/Bcl-2/Bax, and SIRT3/SOD2 expression) among multiple groups were analyzed using ANOVA validated by Tukey’s post hoc test. The t test was utilized to compare differences of variables between 2 groups. A p<0.05 value was considered as statistical significance.

**Figure 1.** Graphs for the distribution of the cell cycles in Sca-1+ HSC/HPC cells (±s, n=6). (A) Distribution of G0/G1 phase. (B) Distribution of G2/M phase. (C) Distribution of S phase. (D) Proliferative index. * p<0.05 compared to Control group, * p<0.05 compared to D-gal aging model group, # p<0.05 compared to Rg1 treatment group.

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of the D-gal aging model group (Table 2, p<0.05). The Rg1 prevention group had significantly higher CFU-Mix colony-formation compared to the Rg1 treatment group (Table 2, p<0.05).

Rg1 reduces the G0/G1 phase of Sca-1+ HSC/HPC cells

Our data indicated that Sca-1+ HSC/HPC in G0/G1 phase in the D-gal aging group was remarkably higher than in the Control group (Figure 1A, p<0.05). The number of Sca-1+ HSC/HPC cells in G2/M phase was remarkably higher (Figure 1B, p<0.05) and the number in S phase was remarkably lower (Figure 1C, p<0.05) compared to the Control group. There were no significant differences in numbers of Sca-1+ HSC/HPC cells in G0/G1 phase between the Rg1 treatment group or Rg1 prevention group and the Control group (Figure 1A, p>0.05). The proliferative index (PI, S+G2/M) in the D-gal aging group was also remarkably lower than in the Control group (Figure 1D, p<0.05). PI was also significantly increased in the Rg1 treatment and prevention group compared to that of the D-gal aging model group (Figure 1D, p<0.05). Moreover, Bax levels in the Rg1 treatment and prevention group were significantly lower than in the D-gal aging model group (Figure 1D, p<0.05).

Rg1 decreased cleaved caspase 3 levels and changed levels of Bcl-2 and Bax

To determine the aging mechanism in Sca-1+ HSC/HPC cells, the apoptosis-associated caspase 3 and downstream molecules, such Bcl-2 and Bax, were evaluated. Our findings demonstrated that cleaved caspase 3 levels were remarkably reduced in the Rg1 treatment group and Rg1 prevention group compared to the D-gal aging model group (Figure 2, both p<0.05). Bcl-2 expressions were significantly enhanced in the Rg1 treatment and prevention group compared to that of the D-gal aging model group (Figure 2, p<0.05). Moreover, Bax levels in the Rg1 treatment and prevention group were significantly lower than in the D-gal aging model group (Figure 2, p<0.05).

Rg1 upregulated SIRT3 and SOD2 mRNA levels

Compared to the Control group, SIRT3 (Figure 3A) and SOD2 (Figure 3B) mRNA levels in the Rg1 Control were significantly upregulated (p<0.05). The SIRT3 (Figure 3A) and SOD2 (Figure 3B) mRNA levels in the D-gal aging model group were obviously lower than in the Control group (p<0.05). Furthermore, SIRT3 (Figure 3A) and SOD2 (Figure 3B) mRNA levels in the Rg1
treatment group and Rg1 prevention group were significantly higher than in the D-gal aging model group (both \( p < 0.05 \)), while the Rg1 prevention group exhibited significantly stronger effects on SIRT3 and SOD2 mRNA expression.

Figure 3. Effects of treatment of Rg1 on the SIRT3 and SOD2 expression in Sca-1\(^+\) HSC/HPC cells (\( \bar{x} \pm s, n=6 \)). (A) Relative mRNA expression of SIRT3. (B) Relative mRNA expression of SOD2. * \( p < 0.05 \) compared with that of Control group, * \( p < 0.05 \) compared to D-gal aging model group, \# \( p < 0.05 \) compared to treatment group.

Figure 4. SIRT3 and SOD2 protein expression in Sca-1\(^+\) HSC/HPC cells (\( \bar{x} \pm s, n=6 \)). (A) Western blot bands for the SIRT3 and SOD2 expression. (B) Statistical analysis for relative SIRT3 protein expression. (C) Statistical analysis for the relative SOD2 expression. * \( p < 0.05 \) compared to Control group, * \( p < 0.05 \) compared with that of D-gal aging model group, \# \( p < 0.05 \) compared to Rg1 treatment group.

Rg1 enhanced expression of SIRT3 and SOD2 in Sca-1\(^+\) HSC/HPC cells

SIRT3 (Figure 4A, 4B) and SOD2 (Figure 4A, 4C) protein expression in the D-gal aging group was remarkably lower than in the Control group (\( p < 0.05 \)), and levels in the Rg1 Control group were remarkably higher than in the Control group (\( p < 0.05 \)). Furthermore, compared the D-gal aging model group, SIRT3 (Figure 4B) and SOD2 (Figure 4C) protein expression levels in...
the Rg1 treatment group and Rg1 prevention group were significantly higher (both p<0.05). Interestingly, we also found that the Rg1 prevention group showed obviously stronger effects on SIRT3 and SOD2 protein expression.

Discussion

Ginseng is an important Chinese herbal drug characterized by the roles of benefitting qi and nourishing blood according to the Traditional Chinese Medicine theory [21]. The modern medical theory [22,23] also illustrates that ginseng is characterized by the functions of anti-injury, anti-aging, anti-oxidant, and enhanced immunity. Zhu et al. [17] also reported that Rg1 could prolong the life of mice and delay aging of human lung fibroblasts.

During the processes of natural aging, the hematopoietic system undergoes progressive functional or morphologic changes, which usually occurs in the HSC/HPC cells [24]. Zhou et al. [25] reported that Rg1 could delay the tert-butyl hydroperoxide (t-BHP)-induced aging of HSCs by signaling pathways regulating p19α, p16\(^{INK4a}\), p21\(^{Cip/Waf1}\), and p53. However, the mechanism that delays aging of HSC/HPC cells has been unclear; therefore, the targeting regulatory molecules that mediate Rg1 delayed aging is now an important research focus [21,26]. Zhu et al. [17] also demonstrated that Rg1 could protect the hippocampus against abnormalities in a rat model of D-gal treatment-induced aging.

ROS accumulation causes aging and age-related disorders [27,28]. D-gal can cause the production of galactitol, which accumulates in cells and results in ROS production undergoing effects of aldose reductase [29]. Long-term treatment with D-gal can induce symptoms of natural aging, such as cognitive dysfunction [30], a shortened lifespan [31], oxidative stress [32], neuro-degeneration [33], and immunologic defects [34]. Therefore, this research used D-gal-induced Sca-1\(^+\) aging rats to determine the effects of Rg1 treatment on aging. We found that Rg1 treatment remarkably reduced the proportion of cells in G0/G1 phase and SA-β-Gal-positive staining Sca-1\(^+\) HSC/HPC cells, and significantly increased the CFU-Mix levels compared to the D-gal model. All of the above results suggest that Rg1 treatment has anti-aging effects on Sca-1\(^+\) HSC/HPC cells and promotes cell proliferation in the aging rat model.

In the present research, we also found that Rg1 could significantly decrease the levels of cleaved caspase 3, which is the activated molecule of caspase 3 and can directly induce cell apoptosis [35]. Rg1 also remarkably decreased the Bax expression in Sca-1\(^+\) HSC/HPC cells, which is a pro-apoptotic molecule [36]. Moreover, Rg1 significantly increased the expression of the anti-apoptotic molecule Bcl-2 in Sca-1\(^+\) HSC/HPC cells [36]. Therefore, the above results suggest that Rg1 can inhibit the aging of Sca-1\(^+\) HSC/HPC cells in an aging rat model by inducing apoptosis.

Metabolism is achieved through the two-way regenerative function of acetylase, which is the key regenerative factor for processing nutrients in the metabolism cycle [37]. SIRT3 is a deacetylase that has a critical role in mitochondria by regulating and eliminating ROS [38,39]. SIRT3 can enhance SOD2 activity and accelerate ROS clearance by triggering deacetylation. SIRT3 also plays an important role in negatively regulating aging. Previous studies [40,41] discovered that SIRT3 can promote the stability of mitochondria and decrease ROS production in normal HSCs. However, in HSCs undergoing stress or in aged HSCs, SIRT3 can promote the self-renewal and multi-differentiation capacity, and further delay aging processes of HSCs [40,42]. The present study demonstrated that SIRT3 and SOD2 were significantly upregulated in the Sca-1\(^+\) HSC/HPC cells of the aging model group compared to that of the D-gal model group, and this is consistent with the changes in SIRT3 and SOD2 in vivo [43]. Meanwhile, SIRT3 and SOD2 expressions were significantly increased in Sca-1\(^+\) HSC/HPC cells of the Rg1 treatment or prevention group compared to that of the D-gal aging model group, which suggests that Rg1 enhances SIRT3 expression in Sca-1\(^+\) HSC/HPC cells, and increased levels of SIRT3 further upregulates the SOD2 activity.

Conclusions

Rg1 decreased the SA-β-Gal-positive staining in Sca-1\(^+\) HSC/HPC cells, and enhanced the colony-formation capacity of CFU-Mix, which suggests that Rg1 delays D-gal-induced aging in a rat model. Rg1 treatment significantly inhibited apoptosis of D-gal-induced aging cells by decreasing cleaved caspase 3 and Bax mRNA expression, and increasing Bcl-2 mRNA expression. SIRT3 and SOD2 levels were significantly increased in Rg1-treated D-gal aging model rats, which suggests that SIRT3 and SOD2 participate in the protective effects of Rg1. Therefore, Rg1 displayed the functions of anti-aging in Sca-1\(^+\) HSC/HPC cells in a D-gal-induced aging rat model by inhibiting mitochondrial pathway-mediated apoptosis and activating the SIRT3/SOD2 signaling pathway. Our results may provide a potential strategy for using Rg1 to inhibit or prevent the aging of Sca-1\(^+\) HSC/HPC cells, which could help to clinically treat aging.

Conflict of interest

None.
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