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Original Article

Protective effect of hydroxysafflor yellow A on MSCs against senescence induced by D-galactose

Xiaoqing Song *, Jinying Wang, Yu Zhang, Xinqian Du, Qibing Qian

Basic Medical College of Hebei North University, Zhangjiakou 075000, China

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ABSTRACT

Objective: To examine the protective effects of hydroxysafflor yellow A (HSYA) against the senescence of mesenchymal stem cells (MSCs) induced by D-galactose (D-gal) *in vitro*, and investigate the potential mechanism involved.

Methods: Grouping experiment, Normal control (NC) group: conventional culture with complete medium; Senescence group: MSCs were cultured for 48 h with complete medium containing 10 g/L D-gal; HSYA group: on the basis of senescence induction, HSYA with the suitable concentration was used to protect MSCs. The key experimental indices associated with oxidative stress, inflammatory response, cell senescence, proliferation and apoptosis were measured through chemical colorimetry, β-galactosidase staining, EdU incorporation and flow cytometry, respectively. The relative quantity (RQ) of proteins related closely to cell proliferation, apoptosis, and NF-κB signaling were measured by Western blotting.

Results: As compared with Senescence group, treatment with HSYA (120 mg/L) effectively ameliorated the adverse situation of MSCs. Oxidation stress and inflammation along with D-Gal induction was dramatically alleviated in MSCs; The β-Gal-positive staining indicated that MSC senescence was significantly mitigated; The proliferative capability of MSCs was significantly increased by up-regulating PCNA and inhibiting p16 expression; The anti-apoptotic effect on MSCs was exerted by down-regulating the RQ of cleaved Caspase-3 and Bax; The activity of NF-κB signaling in MSCs was notably suppressed through inhibiting phosphorylation of IKKβ and p65.

Conclusion: HSYA (120 mg/L) significantly delayed the D-Gal-induced senescence process in MSCs through attenuating inflammatory reaction and oxidative stress, and suppressing the activity of NF-κB signaling.

1. Introduction

As one kind of important adult stem cells, mesenchymal stem cells (MSCs) are effortlessly isolated from connective tissues of human or animals' tissues (Chiang et al., 2016; Ozkan et al., 2018; Wang et al., 2016), and they were demonstrated to display the potent differentiation potentials (Guo et al., 2017; Park et al., 2017). Meanwhile, MSCs was proved to exhibit low expression of major histocompatibility complex I (MHC I), and don't express MHC II molecules, so allogeneic transplantation won't evoke strong immune rejection (Yang et al., 2017). In view of the advantages above mentioned, MSCs are regarded as the "seed" cells in regenerating and repairing injured tissues. Although MSCs were exhibited to exist in a variety of tissues, their ratio in cell population is actually limited. Even through in bone marrow, MSCs only account for 0.001%–0.010% of nucleated cells. So it's essential to amplify MSCs through *in-vitro* culture and therefore obtain a sufficient number of cells. However, the consecutive *in vitro* passages were reportedly vulnerable to lead to MSC senescence (Wang et al., 2016). According to the theory of oxidative damage, senescence is elicited by accumulation of the damage induced by reactive oxygen species (ROS), which are produced during the metabolism process (Lei et al., 2016; Xiang et al., 2021). Excessive ROS will lead to a series of destructive reactions, including lipid peroxidation, protein degradation and DNA breakage, resulting in cell necrosis and ROS leak. What's more, cell senescence was reported to be characteristic by the irreversible arrest of cell cycle and secretion of pro-inflammatory factors: tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and others (Li et al., 2015; Strooper & Arancibia-Cárcamo, 2021). Binding of TNF-α and IL-1β to their respective receptors results in cell apoptosis. Given all of that, cell senescence will remarkably impede the therapeutic effects of MSCs on injured tissues. Therefore, it will be of great significance *in vitro*, to effec-

* Corresponding author.
E-mail address: songxiaoqing0417@163.com (X. Song).

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tively delay the senescent process of MSCs through drug intervention.

As one kind of chalcone glycoside, hydroxysafflor yellow A (HSYA) is mainly extracted from *Carthami Flos* (Honghua in Chinese), and widely used in the prevention and treatment of cardio-cerebro vascular diseases in clinic. Nowadays, a great number of pharmacological researches revealed that HSYA exerted a prominent influence on anti-inflammation, anti-oxidation and inhibition of cell apoptosis. For instance, our previous study under the conditions of hypoxia and serum deprivation *in vitro* confirmed that HSYA treatment dramatically reduced the apoptosis rate of MSCs and enhanced their survival capability by activating HIF-α/VEGF signaling and reducing ROS production in cells (Song et al., 2018). Meanwhile, HSYA was revealed to enhance the repairing effects on tissue traumas in animal models significantly (Wang et al., 2016; Sun et al., 2018; Xu et al., 2017), through reducing the levels of malondialdehyde (MDA) and glutathione disulfide (GSSG), and concomitantly increasing the relative activity of superoxide dismutase (SOD) and catalase (CAT), the level of glutathione (GSH) and the ratio of GSH/GSSG. And accumulated data suggested HSYA administration alleviated inflammatory reaction by down-regulating TNF-α, IL-1β and IL-6 levels in animal models, including strike-induced acute soft tissue injury (Dong et al., 2017), myocardial infarction (Zhou, 2013), brain ischemic reperfusion (Ye & Gao, 2008) and lung injury models (Sun et al., 2010). Prompted by these findings, we hypothesized herein that HSYA could delay the process of cell senescence based on its beneficial pharmacological action of anti-inflammation and anti-oxidation. For confirmation of our hypothesis, HSYA was employed to intervene in MSC senescence induced by D-galactose (D-gal). In cells, D-gal is transformed into galactitol under the catalyzation of aldose reductase. But it is generally deemed that galactitol can't be further metabolized, which further leads to the disorder of intracellular glucose metabolism, and an imbalance between production of ROS and its scavenging. Therefore, D-gal treatment weakens the capability of maintaining steady state in cells, resulting in the irreversible degeneration in function, which accords with the property of cell senescence (Yang & Yi, 2018). Nowadays, D-gal is widely used in establishing the senescence model of cells and animals (Lei et al., 2016; Min et al., 2020; Xiang et al., 2021).

Reportedly, the activated death receptors by TNF-α and IL-1β could initiate NF-κB signal pathway involved in cell senescence (Li et al., 2017). As one transcription factor, NF-κB is composed of five subunits: RelA (p65), RelB (p50), RelC, NFKB1 and NFKB2 in mammalian cells. Normally, NF-κB signaling stays inactive for sequestration by its inhibitory factor κB (IκB) in the cytoplasm. But various adverse stresses, such as inflammation, oxidative stress, and ionizing radiation, can activate the kinase of IκB (IKK), consisting of IKKα and IKKβ, through a series of cascade enzymatic reactions, including phosphorylation. Phosphorylated IKKβ (p-IKKβ) catalyzes the target serine residue of IκB to undergo phosphorylation. Correspondingly, the protein degradation of phosphorylated IκB was executed by ubiquitin–proteasome system, and thereby the released p65 is phosphorylated by p-IKKβ to produce phosphorylated p65 (p-p65). Under the direction of the nuclear location sequence (NLS), p-p65 is translocated into the nucleus, and activates the expression of senescence-related genes (Sivapurapu, 2015; Cai et al., 2015). It is suggested that inhibition of the activity of NF-κB pathway may delay the senescence process of MSCs at the molecular level. During senescence, the functions of MSCs in immunomodulation, secretion of trophic cytokines and tissue repairing capability will become weakened gradually, and therefore their therapeutic effects were undermined in regenerative medicine.

In order to delay MSC senescence and investigate the underlying mechanism, our present study used HSYA to protect MSCs against senescence caused by D-gal induction *in vitro*, thus promoting the clinical application of MSCs. And cell viability and proliferative ability of MSCs, the activity of NF-κB signal pathway and other biochemical indices were detected to authenticate our hypothesis above mentioned, and meanwhile to explore the potential mechanism of HSYA against senescence.

2. Materials and methods

2.1. Culture and identification of MSCs

About 3-week SD male rats were purchased from the medical department of Peking University (SCXK (Jing) 2016–0010), and the experimental procedures met the standards of Animal Ethics Committee of Hebei North University (HBNV20200321021084).

Rats were anesthetized with sodium isopentabarbital (30 mg/kg body weight) via intraperitoneal injection, and their femurs were taken under sterile conditions. After rinse with sterile normal saline, cells were harvested by flushing the femur medullary cavity with complete medium (L-DMEM containing 10% FBS) (Acmec Biotech Co., Ltd., Shanghai, China), and then cell culture was performed through whole bone marrow adherence under the conditions of a 37 °C humidified atmosphere plus 5% CO_2. The medium was replaced every 3 d, during which non-attaching-wall cells were removed gradually. At about 90% confluence, 0.25% trypsin containing 0.01% EDTA was used to digest cell monolayer into single cells for subculture at a ratio of 1:2.

After immunocytochemical staining with the cell-surface antigens, mesenchymal identification of cells cultured was performed. Briefly, MSCs at Passage 4 (P_4) were washed with phosphate buffered saline (PBS, pH = 7.2) for three times, and incubated with 10 μL CD29-PE, CD34-FITC, CD45-FITC and CD90-APC rat-specific monoclonal antibodies (BD Pharmingen, SanDiego, CA, USA) at room temperature for 30 min. Lastly, the immunophenotype of MSCs was determined through flow cytometry (FCM).

2.2. Establishing senescence model of MSCs and HSYA intervention

After authentication of MSCs, grouping experiment with MSCs at P_4 was carried out. Senescence group: MSCs were cultured for 48 h in the complete medium containing 10 g/L D-gal (Yan et al., 2013; Yang & Yi, 2018) to induce cell senescence. Normal control (NC) group: MSCs was cultured using the complete medium. Protection group was further divided into five sub-groups: Based on the senescence induction using D-gal, 40, 80, 120, 160 and 200 mg/L HSYA (Yuanye Biotech Co., Ltd., Shanghai, China) were respectively used to protect MSCs against senescence.

2.3. Detection of cell viability by MTT

According to the grouping method abovementioned, MSCs of 100 μL with a density of 10^5 cells/mL were seeded in 96-well plates. After adherence culture, the medium was replaced with 5 mg/mL MTT (Beyotime Biotech Co., Ltd., Shanghai, China), and 37 °C incubation in cell incubator was maintained for 4 h. Thereafter, MTT medium was removed, and 150 μL DMSO was added in. After full shake at room temperature for 10 min, the optical density (OD) value was read using the microplate reader (λ = 570 nm). According to cell viability measured, the most suitable concentration of HSYA was screened, which was named HSYA group in the downstream experiment.

2.4. Estimation of oxidative stress and inflammation

To estimate the oxidative stress in MSCs of all groups, the relative activity of SOD and the relative content of MDA were respectively measured using the methods of nitro blue tetrazolium (NBT) and thiobarbituric acid (TBA), respectively. The protein concentration of the supernatant samples was detected by bicinchoninic acid (BCA) method. The amount of ROS was determined using the 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) agent. Namely, (1×10^6) MSCs were respectively harvested from three groups, dark incubation with DCFH-DA fluorescent probe was kept for 30 min at room temperature, and then the ROS content in MSCs was evaluated according to the DCF intensity detected by FCM. In addition, for evaluation of inflammatory reaction in MSCs, the amount of TNF-α, IL-1β and IL-4 secreted into medium was determined using enzyme-linked immunosorbent assay (ELISA) kits (Mskbio., Co., ltd., Wuhan, China).

2.5. Senescence-associated β-galactosidase (SA-β-gal) staining

The increase in β-galactosidase activity is one prominent characteristic of cell senescence, so SA-β-gal staining was considered as one of the most direct methods to assess cell senescence (Lee et al., 2006). In this literature, SA-β-gal staining was performed according to the manufacturer's protocols of SA-β-gal staining kits (Shanghai Beyotime Institute of Biotechnology, Co., ltd.). After adherent culture of 0.5 mL MSCs with a density of (5×10^5)/L in 24-well plates, cells were fixed with 4% polyformaldehyde for 15 min at room temperature. Thereafter, 37 °C incubation with SA-β-gal staining solution, containing 5% X-Gal reagent, was maintained overnight in a waterbath. Under the phase contrast microscope (Eclipse90i, Nikon), cells presenting blue color represent the SA-β-gal-positive cells, and the senescent rate was counted.

2.6. EdU (ethyl-2-deoxyuridine) staining

MSCs (100 μL) with (1×10^5)/L density were planted in 96-well plates for induction culture. EdU (KeyGen Biotech. Co. ltd. Nanjing China) solution was added into the culture wells and the final concentration of EdU was 10 μmol/L. After 37 °C incubation in the 5% CO_2 incubator for 24 h MSCs were fixed for 15 min with 4% polyformaldehyde and stained by kFluor488-azide reagent for 15 min at room temperature. Subsequently 0.5 μg/mL Hoechst33342 were employed to re-dye the nuclei for 5 min at room temperature. The images were captured under a fluorescence microscope (Imager M2 Zeiss).

2.7. Detection of cell apoptosis

According to the manufacturer of Annexin V/PI assay kits (Beyotime, Shanghai), cell apoptosis was determined through FCM. In brief, a sum of (1×10^5) MSCs taken respectively from three groups were rinsed with pre-cooled PBS and mixed with 500 μL Binding buffer, followed by addition of 50 μL Annexin V-FITC into the cell suspension. After 4 °C incubation for 20 min in the dark, 200 μL PropidiumIodide (PI) reagent was added, and the dark incubation was kept for 20 min at 4 °C. After rinse with PBS for three times, the apoptotic rate of MSCs was examined using FCM.

2.8. Western blotting

The total protein of MSCs was extracted from three groups, and the protein concentration was detected using BCA method. Samples containing 20 μg protein were mixed with 5 × loading buffer and electrophoresed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, protein bands were electrotransferred onto PVDF membranes. After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (1:5000 dilution; Bioss Biotech Co. ltd., Beijing, China) and HRP-conjugated goat anti-rabbit secondary antibody (1:3000 dilution; ABclonal Co., ltd., Wuhan, China). Finally, electrochemiluminescence (ECL) reagent (Beyotime, Shanghai, China) was used to visualize the protein bands, and the gray values were analyzed with Image J software. The primary antibodies of proteins detected were used in this paper, involving in cell proliferation, proliferating cell nuclear antigen (PCNA) and p16; cell apoptosis, apoptosis effector enzyme--cleaved Caspase-3, and pro-apoptotic protein--Bax; NF-κB signaling, IKKβ and p65. β-actin antibody (1:10,000 dilution; ABclonal) was used as the loading control.

2.9. Statistical analysis

All experiments were conducted at least in triplicate. SPSS17.0 statistical software was applied for data analysis through one-way analysis of variance (ANOVA). All data were expressed by mean ± standard deviation. Values among the groups were considered significantly different at $P < 0.05$.

3. Results

3.1. MSC authentication

To confirm the mesenchymal property of the cells cultured, the cell-surface antigen profiles were determined through FCM. As shown in Fig. 1, MSCs strongly express CD29 and CD90, which were respectively present in (94.13 ± 1.65)% and (84.47 ± 1.15)% of cell population. In comparison, MSCs were weakly positive for CD34 and CD45, with the rates of (2.07 ± 0.25)% and (21.27 ± 2.41)%, respectively. It was deduced that the cultured cells accord with the phenotypic characteristics of MSCs.

3.2. Determination of MSC viability

In MTT assay, OD value indirectly reflects the activity of succinate dehydrogenase in cells, which is one key marker of cell viability (Koma et al., 2019). As shown in Table 1, the viability of MSCs in senescence group was <50% of that in NC group; And based on Senescence induction, OD values were positively correlated with the concentration of HSYA within the range of 40–160 mg/L. It was exhibited that 120 mg/L HSYA could significantly increase MSC viability, which accounted for about 70% of that in NC group. There was a slight increase in cell viability when the concentration of HSYA reached 160 mg/L, but no significant difference existed from 120 mg/L HSYA group. Therefore, 120 mg/L HSYA was screened to protect MSCs against senescence in the downstream experiment.

3.3. Analysis of oxidative stress and inflammatory response

As shown in Fig. 2A–C, D-gal induction notably aggravated the oxidative stress, which was evidenced by the decrease of SOD relative activity, and the increase of MDA and ROS content in MSCs. HSYA treatment effectively ameliorated the adverse situation caused by D-gal induction: the relative activity of SOD increased by about 50% compared with Senescence group, but the contents of MDA and ROS decreased by more than 30%. Meanwhile, ELISA showed as compared with NC group, D-gal led to a substantial increase to three times in secretion of TNF-α and IL-1β into media, and resulted in a slight increase of IL-4. HSYA treatment dramatically mitigated the inflammation in MSCs induced by D-gal

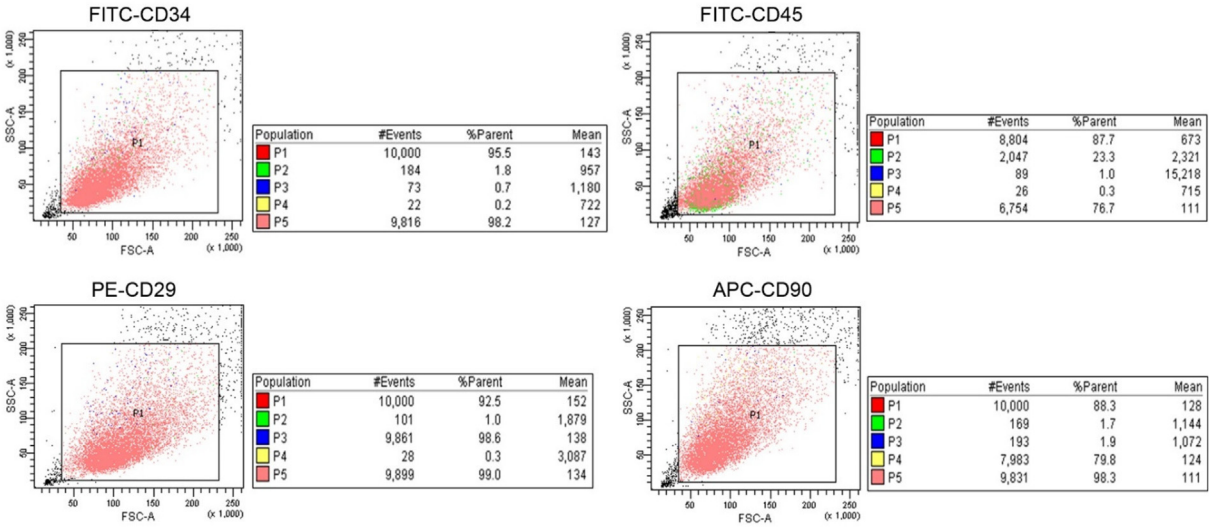

Fig. 1. Determination of MSC surface antigens by flow cytometry (FCM). Cells cultured were strongly positive for CD29 and CD90, but negative for CD34 and CD45, which meets the properties of MSCs. P1, chief cell population; P2, FITC-positive cell population; P3, PE-positive cell population; P4, APC-positive cell population; P5 is a no-load channel.

Table 1
Measurement of relative cell viability (mean ± SD, n = 5).

Groups	OD	Relative viability (%)
NC group	0.516 ± 0.024	100
Senescence group	0.247 ± 0 0.046**	47.89
HSYA (40 mg/L)	0.263 ± 0.046**	50.97
HSYA (80 mg/L)	0.309 ± 0.071**#	59.88
HSYA (120 mg/L)	0.351 ± 0.059**##	68.02
HSYA (160 mg/L)	0.366 ± 0.055**##	70.93
HSYA (200 mg/L)	0.363 ± 0.068**##	70.35

Note: ** means there is a significant difference between NC group and the others (P < 0.01, NC, Normal group); There is a significant difference ($^{\#}P$ < 0.05 and $^{\#\#}P$ < 0.01) *vs* Senescence and HSYA (40 mg/L) groups.

(Fig. 2D–F) through reducing the levels of pro-inflammatory cytokines and raising the amount of anti-inflammatory cytokines.

3.4. Assay of MSC senescence

SA-β-Gal staining (Fig. 3) demonstrated that few β-Gal positive cells was observed in NC group, and the rate was only 3% or so; However, induction with 10 g/L D-gal led the rate of β-Gal positive cells (blue, indicated by the arrow) to reach more than 40%, which was effectively counterturned by the treatment with 120 mg/L HSYA, and the senescent rate of MSCs in HSYA group was only about 25%.

3.5. Assay of MSC proliferation

After 24 h of incubation with EdU reagent, EdU incorporation into nucleus was observed under the fluorescence microscope. The cells whose nuclei present green fluorescence are EdU-labeled and newly proliferated; and the cells carrying blue fluorescent nuclei are hoechst33342-labeled and non-proliferated. As

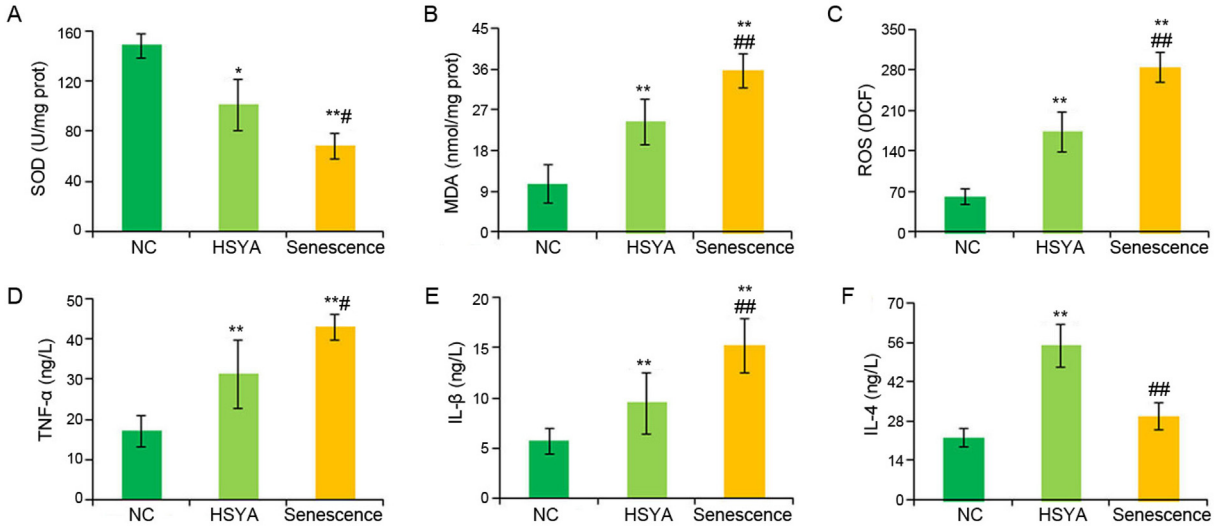

Fig. 2. Assay of oxidative stress and inflammatory response. A–C respectively showed the quantitative analysis of SOD, MDA and ROS; D–F respectively showed the quantitative analysis of TNF-α, IL-1β and IL-4. HSYA treatment dramatically alleviated oxidation stress and inflammatory reaction induced by D-Gal in MSCs ($^{*}P$ < 0.05 and $^{**}P$ < 0.01 *vs* NC group; $^{\#}P$ < 0.05 and $^{\#\#}P$ < 0.01 *vs* HSYA group).

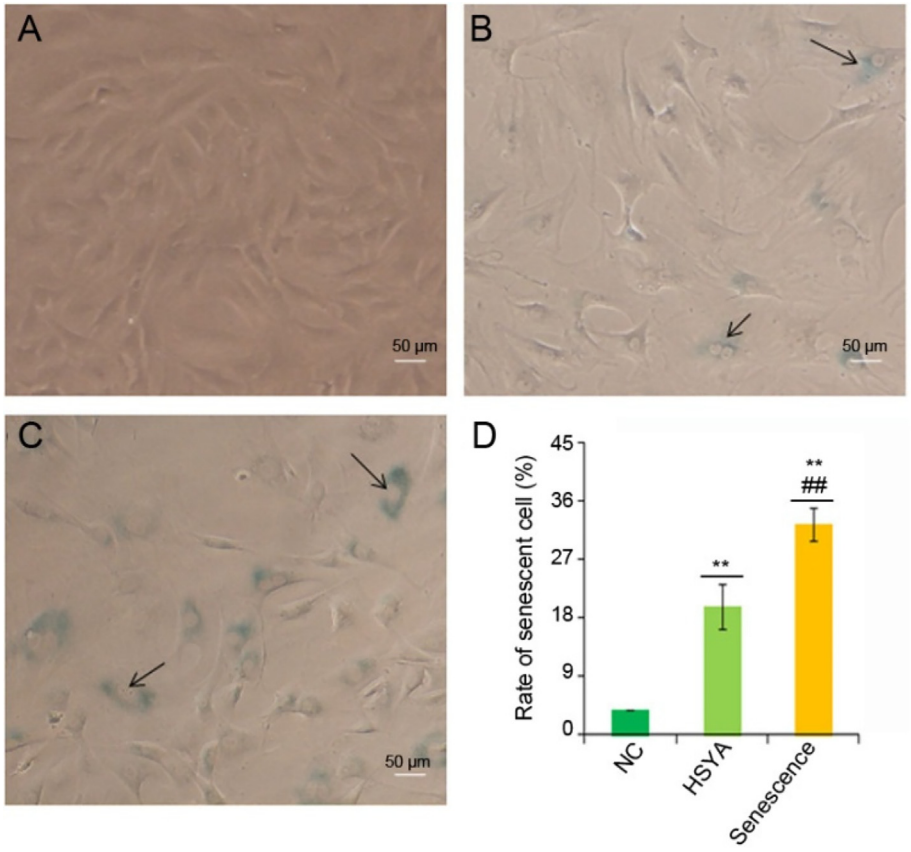

Fig. 3. SA-β-Gal staining of MSCs. A–C respectively exhibited the SA-β-Gal staining results of NC, HSYA and Senescence groups; D. To analyze the rate of senescent MSCs in all groups quantitatively. In contrast with Senescence group, HSYA could reduce the rate of senescent MSCs ($^{**}P < 0.01$ *vs* NC group; $^{\#\#}P < 0.01$ *vs* HSYA group).

presented in Fig. 4A–D: MSCs exposed to D-gal exhibited a significant decrease of EdU-positive cells, and the proliferative rate was only 20% or so in Senescence group; 120 mg/L HSYA treatment notably increased the proportion of EdU-positive cells to about 35%. All in all, although the proliferative rate of MSCs in HSYA group was much lower than that in NC group, but significantly higher than that in Senescence group ($P < 0.01$).

Western blotting confirmed that D-gal induction actually weakened the proliferative ability of MSCs, which was evidenced by the down-regulation of the relative quantity (RQ) of PCNA, a marker of cell proliferation, and the increase in the RQ of p16, an inhibitor of cyclin dependent kinases (CDK) 4 and CDK6. But HSYA intervention was proved to relieve the inhibitory effect of D-gal on MSC proliferation to a certain extent, by enhancing the PCNA expression and suppressing p16 expression.

3.6. Detection of cell apoptosis

According to the result of FCM (Fig. 5A), the apoptotic rate of MSCs in NC, HSYA and Senescence groups was (5.37 ± 0.69)%, (15.16 ± 3.28)% and (25.31 ± 5.17)%, respectively. There was a significant difference between HSYA and Senescence groups, which indicated that HSYA protection significantly abated the apoptotic effect exerted by D-gal treatment. Meanwhile, the inhibitory effect of HSYA against cell apoptosis induced by D-gal was verified through Western blotting. As presented in Fig. 5B–C, the RQ of cleaved Caspase-3 and Bax in HSYA group accounted for about 50% of that in Senescence group, between which the significant difference ($P < 0.01$) existed.

3.7. Analysis of NF-κB signaling activity

In order to elucidate the potential mechanism of HSYA against MSC senescence, the activity of NF-κB signaling was analyzed using Western blotting, and herein the RQ of its key elements: IKKβ and p65 with their phosphorylation levels was detected. As shown in Fig. 6, there was no significant difference in the RQ of IKKβ and p65 among these three groups, but D-gal induction significantly increased their phosphorylation level, which was in consistent with the previous researches that the activity of NF-κB signaling increases significantly in the aging tissues or cells (Tang et al., 2015; Zhang et al., 2016). Compared to Senescence group, 120 mg/L HSYA almost had no inhibitory effect on the expression of IKKβ and p65, but effectively decreased the RQ of p-IKKβ and p-p65, which suggested that HSYA protection could effectively suppressed the activity of NF-κB signaling pathway.

4. Discussion

In view of the potent self-renewal, high multipotential, low immunogenicity and strong "chemotaxis" to the lesion sites, MSCs have attracted great attention as a promising regenerative medicine for many human diseases nowadays. However, cell senescence elicited by successive passage culture *in vitro* reduces the therapeutic effects of MSCs in the tissue repair. How to resist MSC senescence is an imperative event before MSCs are widely used in clinic.

As one reducing acetaldehyde sugar, D-gal has been commonly acknowledged as a senescence-inducing reagent at present. Quite a few studies reportedly applied D-gal to establish the senescent model of MSCs, and thereby explore the senile-resistant method (Yan et al., 2013; Yang & Yi, 2018). In our research, 48 h of induc-

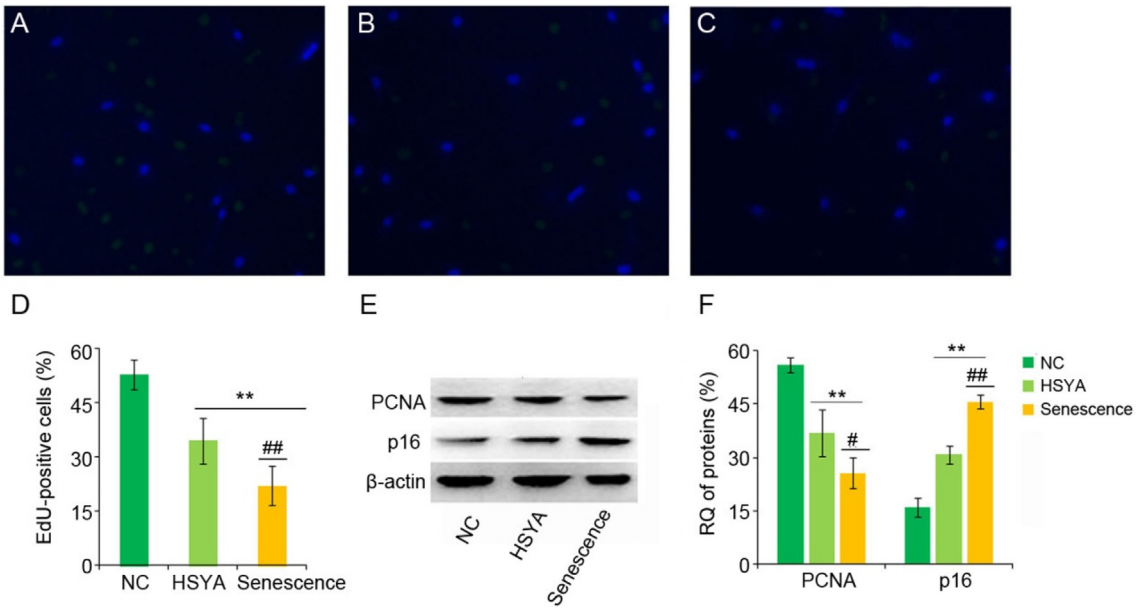

Fig. 4. Proliferative ability of MSCs. A–C respectively showed the results of EdU incorporation in MSCs of NC, HSYA and Senescence groups; D. Quantitative analysis of cell proliferation; E. Western blotting of proteins related to cell proliferation; F. Quantitative analysis of proteins detected. ($^{**}P < 0.01$ *vs* NC group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs* HSYA group).

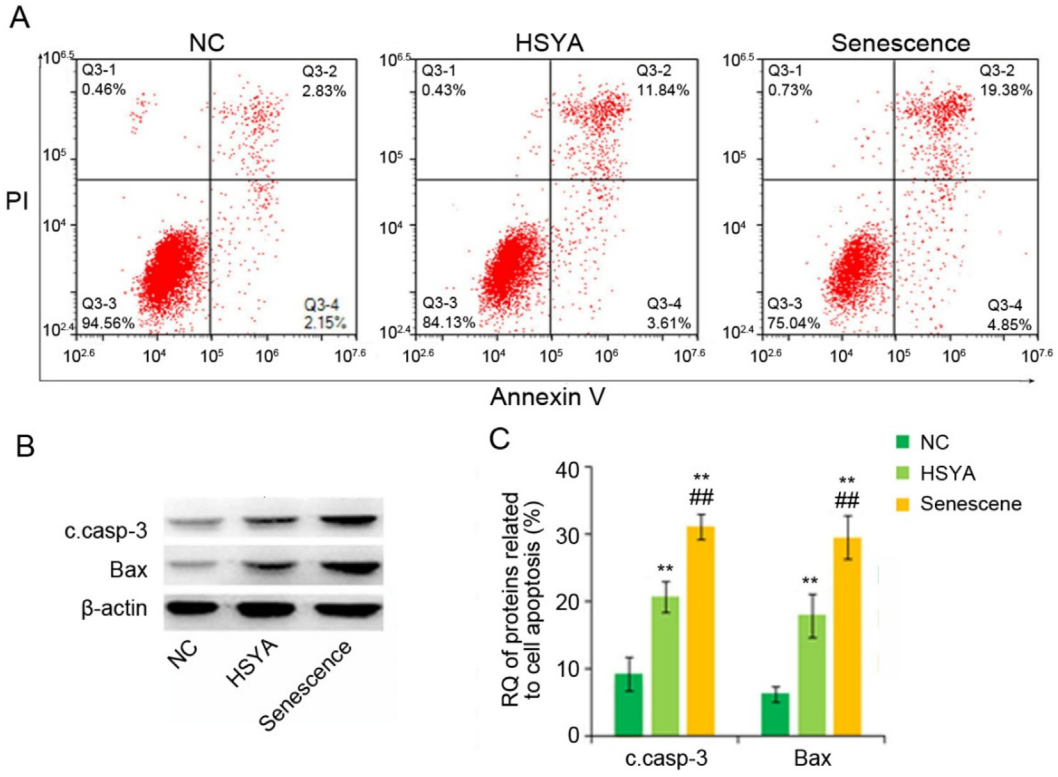

Fig. 5. Analysis of cell apoptosis. A. Detection of MSC apoptosis by flow cytometry; B. Western blotting of apoptosis-associated proteins; C. Quantitative analysis of proteins detected. HSYA protection significantly inhibited cell apoptosis elicited by D-gal treatment through down-regulating the RQ of cleaved Caspase-3 (c.casp-3) and Bax ($^{**}P < 0.01$ *vs* NC group; $^{\#\#}P < 0.01$ *vs* HSYA group).

tion culture with 10 g/L D-gal was revealed to raise the rate of SA-gal-positive cells dramatically, cause the relative activity of SOD to drop sharply, but significantly up-regulate the relative amount of MDA and ROS in MSCs. D-gal exposure was further confirmed to intensify the oxidation stress in cells, which is in agreement with the reports that the senescence-promoting effect of D-gal was exerted by increasing intracellular oxidative stress (Tang et al., 2020; Wang et al., 2019). Meanwhile, our study demonstrated that in comparison with NC group, senescent MSCs secreted abundant pro-inflammatory factors: TNF-α and IL-1β. Inflammatory response has been identified as a hallmark of cell senescence (Chen et al., 2018; Guerrero et al., 2009). Persistent inflammation

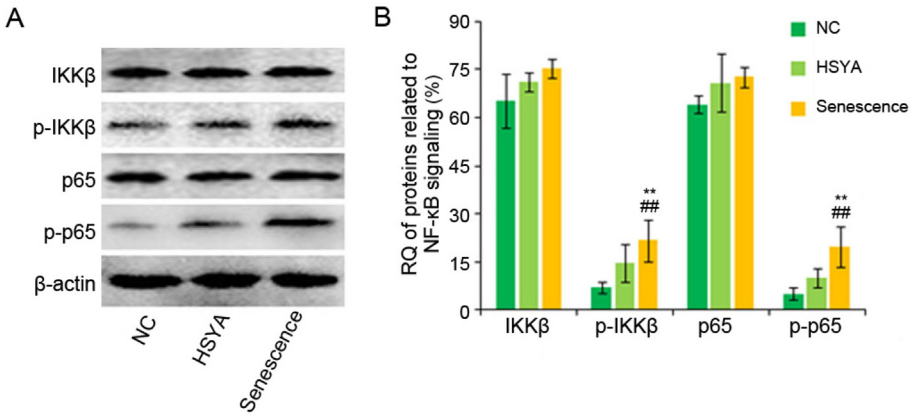

Fig. 6. Assay of NF-κB signaling by Western blotting. A. Western blotting of proteins related to NF-κB signaling; B. Quantitative analysis of proteins detected. Compared to Senescence group, HSYA treatment effectively suppressed the activity of NF-κB pathway by inhibiting phosphorylation of IKKβ and p65. ($^{**}P < 0.01$ *vs* NC group; $^{\#\#}P < 0.01$ *vs* HSYA group).

can activate cell apoptosis mediated by membrane death receptors. HSYA administration was exhibited to markedly alleviate inflammatory response and oxidative stress in this literature. Based on its strong anti-inflammatory and anti-oxidative effects, HSYA was proved in our present study to practically reduce the apoptotic rate of MSCs, further evidenced by the decline in the RQ of cleaved Caspase-3 and Bax. Bax is an important pro-apoptotic protein in Bcl-2 family, and mainly functions in forming a protein channel in the outer membrane of mitochondria, from which cytochrome *C* will be released into the cytoplasm, and thereby the mitochondrial apoptotic pathway is initiated. So HSYA intervention contributed to inhibition of the exogenous and endogenous apoptosis signaling pathway, thus suppressing the caspase cascade reaction of cell apoptosis evoked by D-gal.

Another prominent marker of cell senescence is the decline of cell proliferative capability or even arrest of cell cycle. As a cofactor of DNA polymerase δ, PNCA is considered as the "licensing factor" of DNA replication. PNCA expression commences in G1 phase, and its amount peaks in S phase, so accumulation of PCNA protein in nucleus indicates that the host cell enters S phase or late G1 phase (Perucca et al., 2018). Meantime, the entry from G1 phase into S phase needs the driving force of CDK4 and CDK6. But the activity of CDK4 and CDK6 can be blocked by its inhibitor–p16, which makes cell cycle arrest at G1/S checkpoint. EdU incorporation showed D-gal induction caused the proliferative capability of MSCs to decline steeply. And the results of Western blotting revealed that PCNA protein was notably down-regulated, but the expression of p16 rose dramatically. All the data acknowledged that D-gal induction suppressed MSCs to proliferate, which was in accordance with the property of cell senescence to a certain extent. In contrast with Senescence group, HSYA protection benefited MSC proliferation by up-regulating the expression of PCNA and abating the expression of p16 significantly.

Oxidation-inflammation aging hypothesis is the mainstream theory of cell senescence (Strooper & Arancibia-Cárcamo, 2021). As the molecular "switch" of the inflammatory signaling pathway, the p-p65 subunit was reported to primarily accumulate on the chromatin of senescent cells as compared with that of young counterparts (Chien et al., 2011). NF-κB p65 can activate the expression of inducible nitric oxide synthesis (iNOS), and therefore nitric oxide (NO) is produced. The sustained release of NO exacerbates the inflammatory reaction of cells, which may be the core mechanism of senescence induction (Kriete & Mayo, 2019). So it is indicated suppressing the activity of NF-κB signaling might benefit to delay the senescence process. HSYA intervention in this research didn't exercise significantly promoting effect on the expression of IKKβ and p65, but it could inhibit phosphorylation of IKKβ and p65, so HSYA was suggested to exert an inhibitory influence on activation of NF-κB signaling, which contributed to the delay of MSC senescence.

In conclusion, HSYA treatment significantly improved the viability and proliferative ability of MSCs, reduced the apoptotic rate of MSCs and successfully respited the senescence process of MSCs induced by D-gal, which may have close relationship with its potent effects of anti-inflammation and anti-oxidation, and its inhibition of NF-κB signaling. Therefore, HSYA was revealed to have a protective effect on MSCs against the senescence, and it might be a promising adjuvant drug in MSCs-based therapy for the tissue engineering.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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