



Icariin protects bone marrow mesenchymal stem cells in aplastic anemia by targeting MAPK pathway

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Abstract

Background Icariin, the main pharmacological active flavonoid extracted from *Epimedi herba*, can regulate cellular processes in diverse diseases. The aim of this study was to explore the effects and mechanisms of icariin on proliferation and adipogenesis of bone marrow mesenchymal stem cells (BMSCs) in aplastic anemia (AA).

Methods and results Bone marrow mesenchymal stem cells were isolated from posterior tibias and femurs of AA rats that were induced by benzene and cyclophosphamide and gavaged with icariin. The isolated BMSCs were characterized morphologically and immunologically by positive markers (CD29 and CD90) and negative markers (CD34 and CD45). CCK-8 assay was performed to examine the BMSCs proliferation. Cell apoptosis and cell cycle were detected by flow cytometry. Oil red O staining was carried out to evaluate the adipogenesis of BMSCs. The mRNA expression of PPAR γ , C/EBP- α , and FABP4 was measured by qRT-PCR. The protein levels of p-p38/p38, p-JNK/JNK, p-ERK/ERK, PPAR γ , C/EBP- α , and FABP4 were detected using Western blotting. Icariin promoted the proliferation of BMSCs from AA rats in a dose-dependent manner. The protein levels of p-p38/p38, p-JNK/JNK, and p-ERK/ERK were downregulated in BMSCs from AA rats after icariin treatment. Icariin inhibited the apoptosis and arrested cell cycle at G/S phase of BMSCs from AA rats. The adipogenesis of BMSCs from AA rats was also suppressed after icariin treatment. However, the effects of icariin on BMSCs were weakened by p38 agonist addition.

Conclusions Icariin promoted the proliferation and inhibited the apoptosis and adipogenesis of BMSCs in AA by suppressing MAPK pathway.

Keywords Icariin · Aplastic anemia · Bone marrow mesenchymal stem cells · Adipogenesis · Mitogen-activated protein kinase pathway

Introduction

Aplastic anemia (AA) is a rare and life-threatening bone marrow failure characterized by fatty bone marrow and poor hematopoietic function [1]. The decrease of hematopoietic function can lead to the genitourinary system disorder, intracranial hemorrhage, sepsis, etc., affecting normal life

and work of patients [2]. Currently, the major therapeutic strategies for AA are hematopoietic stem cell transplantation (HSCT) and immunosuppressive therapy (IST) [3]. However, it is difficult to find a matched donor for HSCT, and some lymphocytotoxic agents used in IST may cause complications, such as infections, anaphylaxis fever, and subsequent malignant conditions [4–6]. Therefore, it is urgent to find promising drugs with low side-effects for AA treatment.

The changes of bone marrow hematopoietic microenvironment are closely associated with the pathogenesis of AA [7]. This microenvironment consists of diverse cells, including bone marrow mesenchymal stem cells (BMSCs) [8]. BMSCs act as a critical role in AA progression due to its multi-differentiation ability, which can differentiate to osteoblasts, chondrocytes, adipocytes, etc. [9]. Numerous studies have reported that BMSCs from AA patients appear proliferative defects and the enhanced adipocyte differentiation

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[10–12]. Luo et al. found that the proliferation capacity of BMSCs was reduced and adipocytes were increased in AA rats [11]. Li et al. also denoted that the adipogenic differentiation of BMSCs from children with AA was potentiated [12]. Therefore, finding effective drugs that can protect the proliferation and restrain the adipocyte differentiation of BMSCs in AA is of paramount importance.

Icariin is the main flavonoid constituent extracted from *Epimedi herba* [13]. It has been reported that icariin can affect the proliferation, apoptosis, and differentiation of BMSCs [14–16]. For instance, icariin can promote the proliferation of BMSCs from rats with fractures [14]. Icariin also exhibits the effect of inhibiting adipogenic differentiation of BMSCs [15]. However, the effects of icariin on the growth and differentiation of BMSC in AA remain unclear.

Mitogen-activated protein kinase (MAPK) pathway is an intracellular signaling pathway closely involved in the regulation of cell proliferation and differentiation [17]. Several previous studies indicate that MAPK pathway is related to the adipogenic differentiation of BMSCs [18–20]. Cho et al. demonstrated that BMP-2 induced the adipogenic differentiation of human BMSCs via upregulating MAPK pathway [18]. Zhao et al. indicated that MAPK pathway is activated during the adipogenic differentiation of BMSCs [19]. However, it is still illusive whether icariin can inhibit the adipogenesis of BMSCs in AA via affecting MAPK pathway.

In the present study, the effects of icariin on BMSCs isolated from AA rats were investigated by evaluating the proliferation, apoptosis, and adipogenesis of BMSCs. Moreover, the potential mechanism of icariin against adipogenesis of BMSCs in AA involving in MAPK pathway was uncovered. These findings provide a potential therapeutic drug for AA treatment and offer insights into the investigation of underlying mechanisms.

Materials and methods

Experimental animals and treatments

Animal experiments were carried out in compliance with the guidelines of the Animal Ethics Committee of Zhejiang Chinese Medical University Laboratory Animal Research Center. Clean-grade Sprague–Dawley (SD) rats ($n = 54$, 6 weeks old, weighing 200–250 g) were obtained from the Animal Centre of the Academy of Military Medical Sciences (Beijing, China) and maintained in a temperature-controlled room (24 ± 1 °C) with a 12 h/12 h light/dark schedule. The AA rat model was established by subcutaneously injecting 1 mL/kg benzene per two days for one week and then intraperitoneally injecting 25 mg/kg cyclophosphamide per two days for three times for two weeks. AA model rats were gavaged with a 1:1 mix of normal saline and dimethyl sulfoxide

(DMSO) (the solvent of icariin), different doses of icariin (0.1, 0.5, 1, 5, and 10 μ M), and/or 10 μ M icariin plus p38 agonist (P79350; Invitrogen, Carlsbad, CA, USA). SD rats in control group were treated with physiological saline. After treatment for three weeks, all rats were sacrificed by 3% 150 mg/kg pentobarbital sodium and disinfected with 75% ethanol for isolating BMSCs.

Isolation and characterization of BMSCs

BMSCs were isolated from the femurs and tibias of SD rats. Briefly, femurs and tibias were removed from SD rats and washed with phosphate-buffered saline (PBS) three times in a sterile environment. BMSCs were flushed from posterior tibias and femurs with PBS and then cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 1% L-glutamine. Third generation well-grown cells were harvested and digested with 0.25% trypsin. After centrifuging for 5 min at 4 °C and 1000 rpm, cells were resuspended and identified under a light microscope (Olympus, Japan).

BMSCs isolated from AA rats were analyzed for the presence of MSCs specific markers (CD29, CD34, CD45, and CD90) by flow cytometry. In brief, BMSCs were washed with PBS and blocked with 2% bovine serum albumin (BSA). Then, cells were incubated with primary antibodies against CD29, CD34, CD45, and CD90 for 2 h, followed by the secondary antibody (Alexa fluor 546). Finally, BMSCs were analyzed using a flow cytometer (BD Biosciences, NJ, USA).

Cell proliferation assay

BMSCs suspension was inoculated into a 96-well plate (1×10^3 cells/well) and cultured for 24, 48, and 72 h at 37 °C. Then, 10 μ L CCK-8 solution (Beyotime, China) was added to the wells to incubating for 2 h. Subsequently, the absorbances were measured at 450 nm using a Varioskan™ LUX multimode microplate reader (Thermo scientific, CA, USA).

Flow cytometry

BMSCs cells treated with icariin for 24 h were resuspended in 100 μ L binding buffer to 1×10^6 cells/mL. The apoptosis of BMSCs was detected using an Annexin V/propidium iodide (PI) apoptosis detection kit (KeyGen Biotech, China). Cells were stained with 10 μ L fluorescein isothiocyanate (FITC)-Annexin V and 5 μ L PI under darkness. For cell cycle detection, cells were fixed in 70% ethanol for 12 h at 4 °C. Then, fixed cells were incubated with 10 μ g/mL PI and 100 μ g/mL RNase for 30 min at 37 °C in the dark. Finally, apoptosis and cell cycle condition were detected using a flow

cytometer with CellQuest software (BD Biosciences, NJ, USA).

Adipocyte differentiation and oil red O staining

BMSCs were seeded in six-well plates (4×10^4 cells/well) and cultured in adipogenic-induction medium (high sugar Dulbecco's Modified Eagle Medium (DMEM) supplemented with dexamethasone 10⁻⁸ M, B-glycerin, and ascorbic acid). After 20 days of adipocyte differentiation, cells were fixed with 4% paraformaldehyde and then stained with oil red O (Cyagen Biosciences, China). Finally, the lipid droplet formation was observed under a microscope (Olympus, Japan).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from BMSCs using TRIzol reagent (Invitrogen, CA, USA). cDNA was synthesized using a PrimeScript RT reagent kit (Tiangen, China). qRT-PCR was performed using the SYBR[®] Green reagent (Roche, IN, USA) in a Roche LightCycler[®] 96 System. The reaction program of qRT-PCR was 95 °C for 2 min, and 40 cycles of 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 20 s. The primers were presented in Table 1. The relative mRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western blotting

Total protein of BMSCs was extracted by lysing in ice-cold lysis buffer with protease inhibitor cocktail (Sigma, CA, USA) for 30 min. The protein lysates were separated through SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). After incubating with blocking buffer, membranes were incubated with primary antibodies, including anti-p38, p-p38, JNK, p-JNK, ERK, p-ERK, PPAR γ , C/EBP- α , FABP4, and GAPDH (1:500, CST, MA, USA), at 4 °C overnight. Followed by that, membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (1:1000, CST) for 1 h. Protein images were visualized using an enhanced

chemiluminescence detection kit (Solarbio, China) and captured using the ChemiDoc Imaging System (Bio-Rad, CA, USA). GAPDH was used as the loading control.

Statistical analysis

Each experiment was performed in triplicate. All data were presented as the mean \pm standard deviation. Statistical analyses were performed using SPSS 27.0 (IBM, NY, USA). Statistically significant differences were calculated using one-way analysis of variance followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Results

Morphological characterization of BMSCs in AA model

BMSCs were isolated from the tibia and femur of rats to identify the morphological characteristics of AA and normal tissues. BMSCs presented shorter spindle-shaped morphology and arranged irregularly than normal cells. Moreover, the vacuoles were increased in BMSCs from AA tissues compared with that from control tissues (Fig. 1A). Further, the isolated BMSCs from AA rats showed the positive expression of MSC biomarkers CD29 and CD90, and the negative expression of CD34 and CD45 (Fig. 1B).

Icariin promotes the proliferation of BMSCs in AA

To investigate the effect of icariin on BMSCs in AA, the proliferation of BMSCs were evaluated. As shown in Fig. 2, the proliferation ability of BMSCs from AA model was significantly declined after cultured for 48 h and 72 h compared with that from control ($P < 0.001$). Icariin treatment rescued the decreased proliferation of BMSCs from AA model in a dose-dependent manner. In particular, when treated with 10 μ M icariin, the proliferation of BMSCs was significantly increased ($P < 0.01$) (Fig. 2). Therefore, 10 μ M icariin was used to treat BMSCs in the subsequent experiments.

Table 1 Primer sequences for quantitative real-time PCR

Accession no	Gene name	Primer
NM_013124.3	PPAR γ	Forward: 5'-AGCCCAAGTTTGAGTTTGCT-3' Reverse: 5'-ATTTTCTGGAGCAGCTTGGC-3'
AB020756.1	C/EBP- α	Forward: 5'-ATTTTCTGGAGCAGCTTGGC-3' Reverse: 5'-ATTTTCTGGAGCAGCTTGGC-3'
NM_053365.3	FABP4	Forward: 5'-CGAGATTTTCCTTCAAACCTGGG-3' Reverse: 5'-TCTTGTAGAAGTACGCCTTTC-3'
NM_017008.4	GAPDH	Forward: 5'-CCC CGAGTACAACCTTCTT-3' Reverse: 5'-CGCAGCGATATCGTCATCCA-3'

Fig. 1 Characterization of bone marrow mesenchymal stem cells (BMSCs) **A** The morphology of BMSCs isolated from normal and AA rats. Scale bar = 25 μ m. **B** The expression of MSC specific markers (CD29, CD34, CD45, and CD90) in BMSCs isolated from AA rats

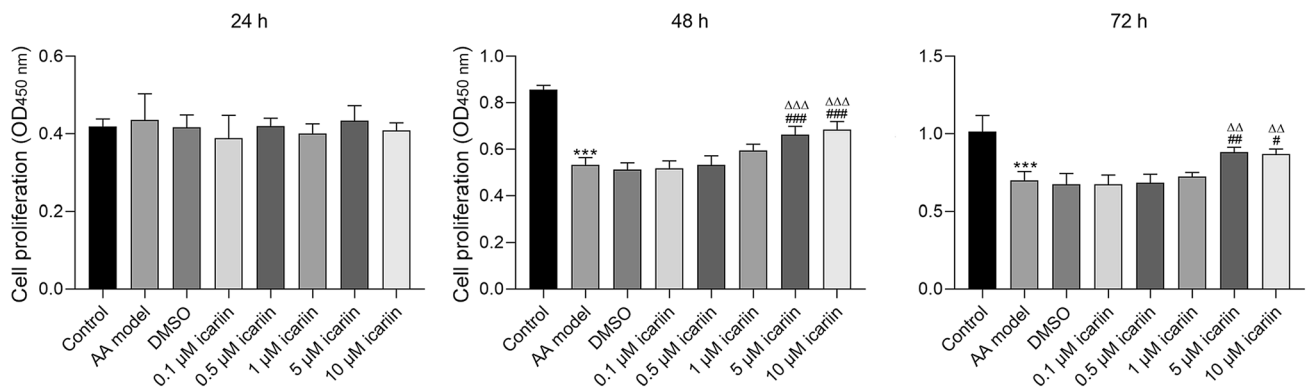
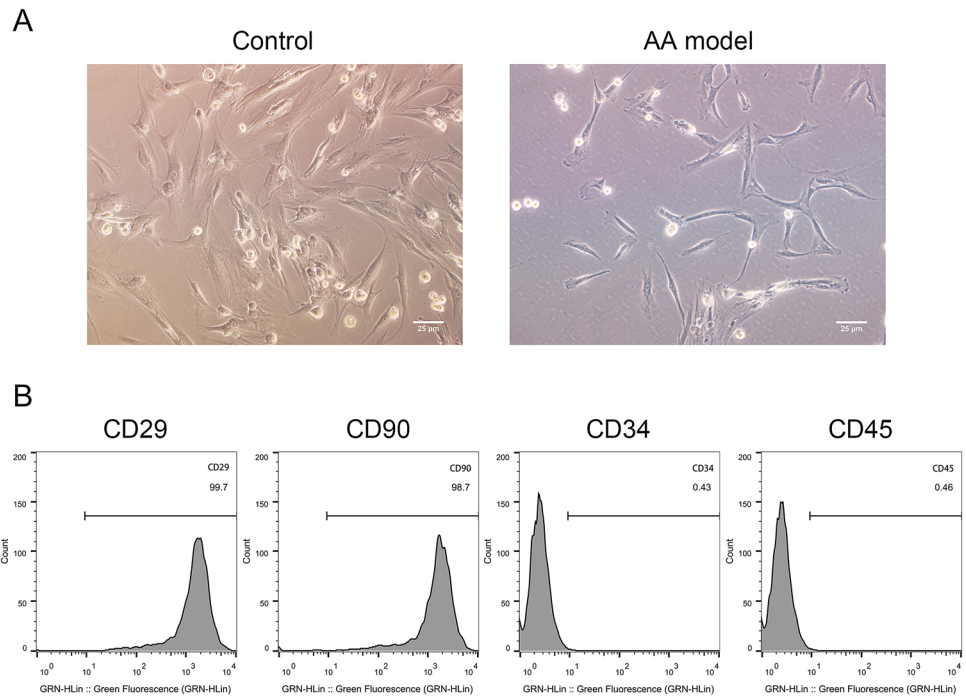


Fig. 2 Icariin dose-dependently promotes the BMSCs proliferation in AA The proliferation of BMSCs from AA rats was measured by CCK-8 assay. AA rats (n=6 per group) were treated with different doses of icariin (0.1, 0.5, 1, 5, 10 μ m) for 24, 48, and 72 h.

*** P <0.001 vs. the control; # P <0.05, ## P <0.01, and ### P <0.001 vs. the AA model; $\Delta\Delta P$ <0.01 and $\Delta\Delta\Delta P$ <0.001 vs. the 0.1 μ m icariin treatment

Icariin inhibits the MAPK signaling pathway in BMSCs from AA model

It has been reported that MAPK signaling pathway is involved in regulating BMSCs proliferation and differentiation [21]. p38, JNK, and ERK are three major protein kinases of MAPK pathway [22]. Our results showed that the protein expression of phosphor (p)-p38/p38, p-JNK/JNK, and p-ERK/ERK in BMSCs from AA model were significantly up-regulated compared with that from control (P <0.001). Icariin administration down-regulated the

expression of p-p38/p38, p-JNK/JNK, and p-ERK/ERK in BMSCs from AA model, whereas p38 agonist weakened the effect of icariin (Fig. 3).

Icariin suppresses the apoptosis of BMSCs from AA model via inhibiting MAPK signaling pathway

To further confirm the promotive effect of icariin on BMSCs proliferation in AA, the apoptosis of BMSCs was determined. Flow cytometry showed that the apoptosis of BMSCs was significantly increased in the AA model

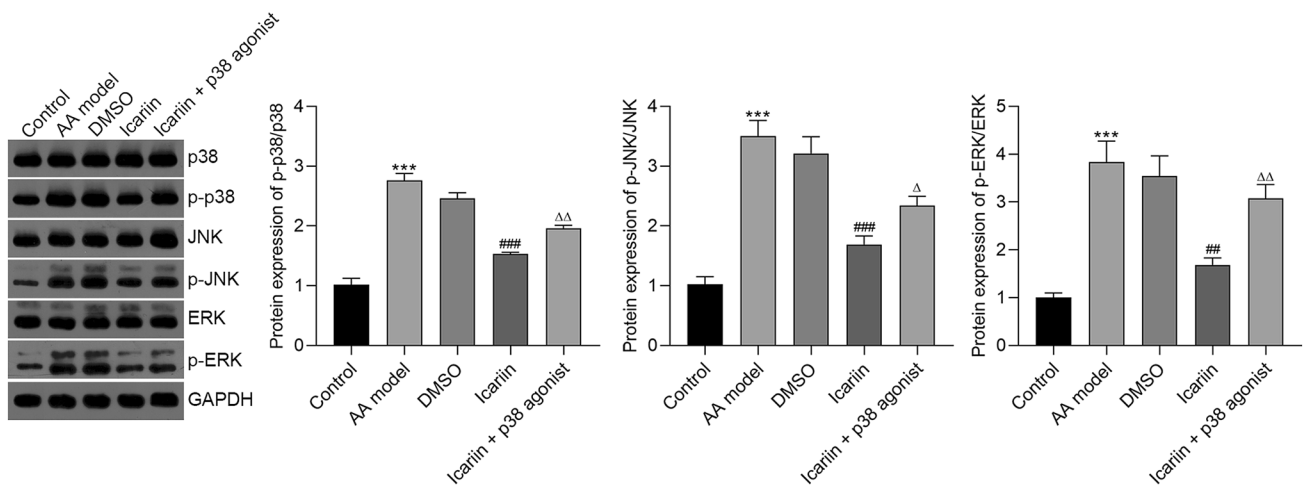


Fig. 3 Icaritin inhibits the MAPK pathway in BMSCs of AA The relative protein levels of p-p38/p38, p-JNK/JNK, and p-ERK/ERK were determined by Western blotting. AA rats (n=6 per group) were treated with 10 μm icaritin and/or p38 agonist. *** $P < 0.001$ vs. the control; ## $P < 0.01$ and ### $P < 0.001$ vs. the AA model; Δ $P < 0.05$ and ΔΔ $P < 0.01$ vs. the icaritin treatment

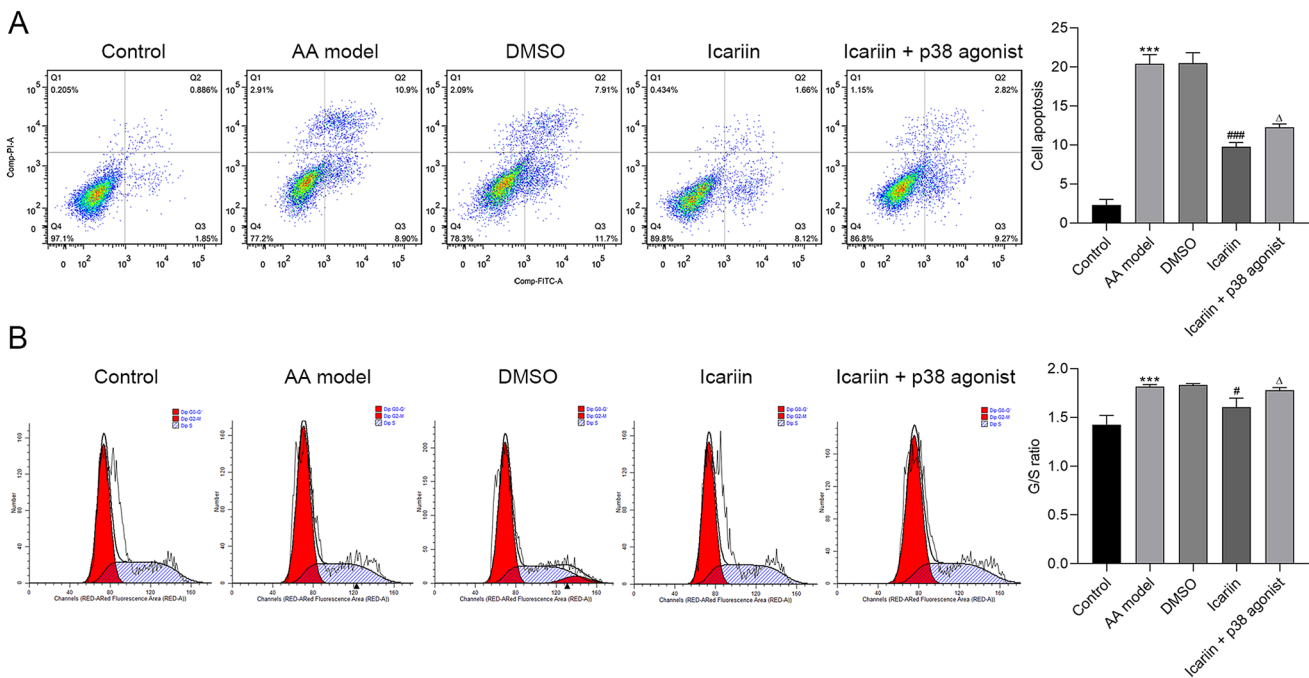


Fig. 4 Icaritin suppresses the apoptosis of BMSCs in AA via inhibiting MAPK pathway **A** The apoptosis of BMSCs from AA rats was measured by flow cytometry. **B** The cell cycle of BMSCs from AA rats was measured by flow cytometry. AA rats (n=6 per group) were treated with 10 μm icaritin and/or p38 agonist. *** $P < 0.001$ vs. the control; # $P < 0.05$ and ### $P < 0.001$ vs. the AA model; Δ $P < 0.05$ vs. the icaritin treatment

compared to that in the control ($P < 0.001$). Icaritin treatment dramatically decreased the apoptosis of BMSCs in the AA model ($P < 0.001$) (Fig. 4A). In addition, the cell cycle phase ratio was also evaluated. BMSCs from AA model presented an increased ratio of G/S phase in comparison to the control ($P < 0.001$). After icaritin treatment, the ratio of G/S phase was dramatically reduced in BMSCs

from AA model ($P < 0.05$) (Fig. 4B). However, p38 agonist partially offset the effects of icaritin on inhibiting the apoptosis and arresting cell cycle of BMSCs from AA rats ($P < 0.05$) (Fig. 4A, B).

Icariin inhibits adipogenic differentiation of BMSCs in AA via retarding MAPK pathway

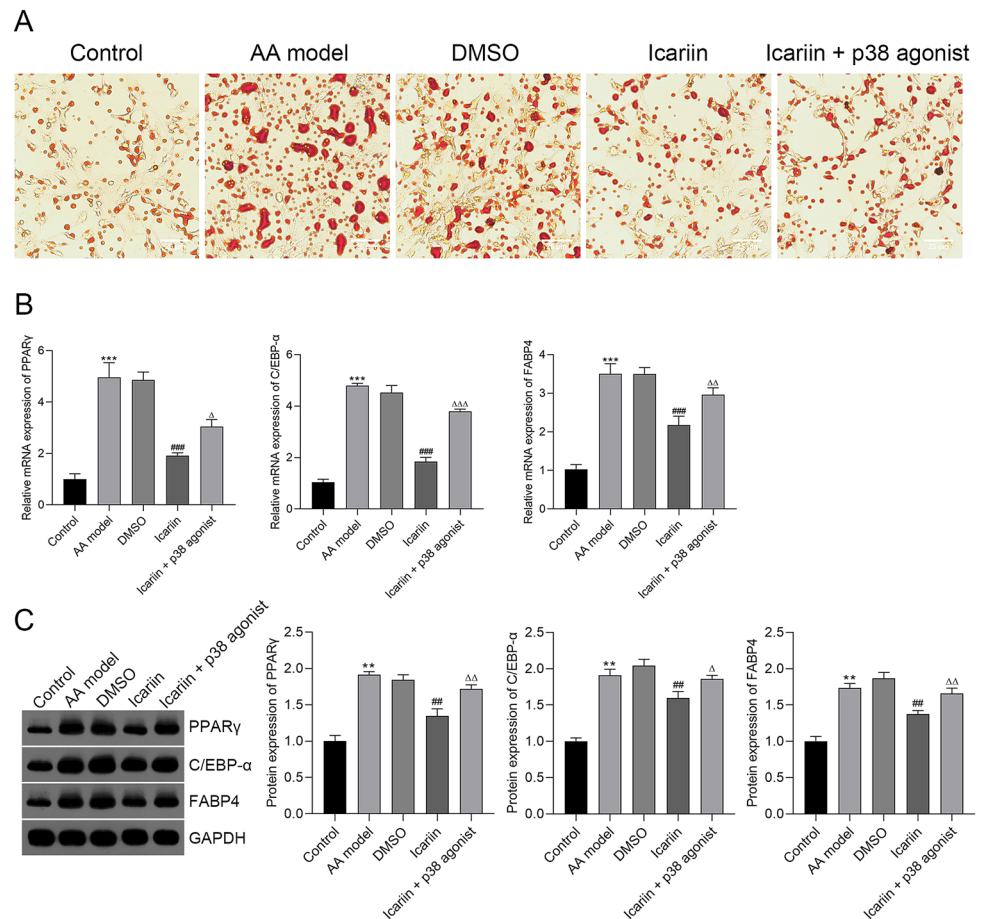
AA is manifested as the decreased hematopoietic function of bone marrow, mainly caused by adipocyte differentiation of BMSCs. Oil Red O staining showed that lipid droplets increased in BMSCs from AA model compared with that from control. Icariin inhibited the lipid droplet formation in BMSCs from AA model, whereas p38 agonist weakened the repressive effect of icariin (Fig. 5A). Moreover, PPAR γ , C/EBP- α , and FABP4 are three adipogenic biomarkers closely related to adipocyte differentiation [23]. We found that the mRNA and protein expression of PPAR γ , C/EBP- α , and FABP4 was significantly up-regulated in BMSCs from AA model compared to that from control ($P < 0.01$). Icariin down-regulated the expression of PPAR γ , C/EBP- α , and FABP4 in BMSCs from AA model, whereas p38 agonist alleviated the effect of icariin ($P < 0.05$) (Fig. 5B, C).

Discussion

AA is a bone marrow dysplasia disease induced by hematopoietic progenitor cell damage [24]. The adipogenic differentiation of BMSCs is one of the major pathological characteristics of AA [25]. Icariin, a flavonoid extracted from *Epimedi herba*, possesses the ability of affecting BMSC adipogenesis [26]. In this study, we found that icariin promoted the proliferation, and inhibited apoptosis and adipogenic differentiation of BMSCs from AA rats. Moreover, the effects of icariin on BMSCs proliferation and adipogenesis in AA were regulated via the inhibition of MAPK signaling pathway.

BMSCs play an essential role in bone marrow hematopoietic microenvironment that is involved in the pathogenesis of AA [8, 11]. AA is characterized by hypoplasia and pancytopenia with increasing fat cells in the bone marrow. BMSCs from AA are more susceptible to be induced into adipogenic differentiation, therefore, adipogenesis has become a critical feature of AA [27]. Previous studies have reported that BMSCs in AA present the decreased proliferation and the increased adipogenesis [28]. Luo et al. found that BMSCs from AA rats manifested as poor proliferation

Fig. 5 Icariin retards the adipogenic differentiation of BMSCs in AA via inhibiting MAPK pathway **A** The mature adipocytes differentiated from BMSCs of AA rats were visualized using oil red O staining. Scale bar = 25 μ m. **B** The relative mRNA expression of PPAR γ , C/EBP α , and FABP4 was measured by qRT-PCR. **C** The relative protein levels of PPAR γ , C/EBP- α , and FABP4 were determined by Western blotting. AA rats (n = 6 per group) were treated with 10 μ m icariin and/or p38 agonist. ** $P < 0.01$ and *** $P < 0.001$ vs. the control; ## $P < 0.01$ and ### $P < 0.001$ vs. the AA model; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, and $\Delta\Delta\Delta P < 0.001$ vs. the icariin treatment



behavior and enhanced adipogenic differentiation [11]. Li et al. revealed that BMSCs from children with AA displayed a better adipogenic differentiation capacity [12]. Consistent with previous studies, we observed the reduced proliferation, and the increased apoptosis and adipogenesis in BMSCs isolated from AA rats after. These results suggest that BMSCs growth is inhibited, whereas its adipogenic differentiation is potentiated in AA.

Icariin is the major flavonoid constituent from *Epimedi herba*, which has been reported to affect BMSC proliferation and differentiation [29, 30]. A study conducted by Wu et al. showed that icariin dose-dependently promoted the proliferation of BMSCs [30]. Similarly, our study found that icariin exhibited a dose-dependent effect on facilitating the proliferation ability of BMSCs from AA rats. Icariin also reduced the apoptosis and cell cycle G/S ratio of BMSCs from AA rats, indicating that icariin has the efficacy of promoting BMSC proliferation in AA. In addition, some previous studies denoted that icariin can suppress the adipogenic differentiation of BMSCs [15, 31]. For instance, icariin inhibited the BMSCs differentiation into adipocyte in ovariectomized mice [15, 31]. In the current study, oil red O staining showed that oil droplet deposition was increased in BMSCs from AA rats after icariin treatment. PPAR γ and C/EBP α are coordinated to control adipogenic differentiation, and FABP4 regulated late adipogenesis [32, 33]. Icariin also downregulated the expression of PPAR γ , C/EBP- α , and FABP4 in BMSCs of AA rats. These results demonstrate that icariin exerts an anti-adipogenic effect on BMSCs in AA.

MAPK signaling pathway acts as a critical role in cell proliferation, apoptosis, and differentiation [34]. So far, several publications have reported that MAPK pathway is involved in the regulation of BMSCs proliferation and adipogenic differentiation [18, 35]. Li et al. unveiled that the activation of MAPK pathway can restrain the proliferation of BMSCs [35]. Cho et al. suggested that the enhanced adipogenic differentiation of BMSCs is modulated by the upregulating of MAPK pathway [18]. Therefore, we speculated that the effects of icariin on BMSCs in AA may be regulated by MAPK signaling pathway. p38, JNK, and ERK are three major members of MAPK pathway [34]. As expected, our study found that icariin downregulated the protein levels of p-p38/p38, p-JNK/JNK, and p-ERK/ERK in BMSCs from AA rats. However, p38 agonist weakened the inhibitory effects of icariin on the expression of p-p38/p38, p-JNK/JNK, and p-ERK/ERK, meanwhile, p38 antagonist enhanced the effects of icariin (Supplementary Fig. 1B). These results indicate that icariin can retard the activation of MAPK pathway in BMSCs derived from AA. To further confirmed whether icariin promotes the proliferation and suppresses the adipogenesis of BMSCs via downregulating MAPK pathway, AA rats were treated with icariin plus p38 agonist. Our results showed that p38 agonist diminished

the inhibitory effects of icariin on the apoptosis, cell cycle G/S arrest, and adipogenic differentiation of BMSCs in AA. Meanwhile, we found that p38 antagonist potentiated the protective effect of icariin on BMSC proliferation in AA (Supplementary Fig. 1A). These findings demonstrate that icariin facilitates the proliferation and suppresses the adipogenesis of BMSCs in AA via deactivating MAPK pathway.

In conclusion, icariin is a promising therapeutic drug for AA via promoting BMSCs proliferation and adipogenic differentiation. The action mechanism of icariin on BMSCs in AA is involved in the downregulation of MAPK pathway. However, our results also showed that there was an increase in early apoptosis in BMSCs of AA rats treated with icariin or/and p38 agonist, thus, the therapeutic effect of icariin on AA is needed to be further evaluated in vivo and in clinical. The underlying mechanism of icariin on BMSCs in AA should be investigated in more depth. Our findings may provide an effective drug for AA treatment and reveal the potential therapeutic target.

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Data availability The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of The First Affiliated Hospital of Zhejiang Chinese Medical University.

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