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Original Article

Curcumin protects mesenchymal stem cells against oxidative stress-induced apoptosis via Akt/mTOR/p70S6K pathway

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Abstract: The application of mesenchymal stem cells (MSCs) for the treatment of ischemic diseases is promising. However, the poor survival of transplanted MSCs has hindered their therapeutic efficacy. Oxidative stress-induced apoptosis is one of the major reasons for their poor survival, while curcumin has the ability to protect cells against oxidative damage. Therefore, we evaluated the effects of curcumin on the survival of MSCs exposed to oxidative stress, and explored its underlying mechanism. Rat bone marrow derived MSCs (BM-MSCs) were treated with various concentrations of hydrogen peroxide (H_2O_2) or curcumin, or they were exposed to H_2O_2 after being pre-treated with different concentrations of curcumin for 3 hours. Cell viability was analyzed by ATP assay, flow cytometry and Hoechst 33258 staining. The expression of apoptosis-related proteins and key proteins in the Akt/mTOR/p70S6K pathway were detected by Western blot. Our results showed that H_2O_2 inhibited the proliferation and survival of BM-MSCs in a concentration- and time-dependent manner. H_2O_2 induced an obvious increase in ratio of Bax/Bcl-2 and expression levels of cleaved caspase-9 and -3. Delightedly, these apoptotic processes could be inhibited or reversed by a pre-incubation with curcumin. We further revealed that the protective effect of curcumin against oxidative injury in BM-MSCs, which could be abolished by Akt inhibitor perifosine, was mediated by elevated phosphorylation of Akt, mTOR and p70S6K. Our data suggest that curcumin is a promising candidate for improving the efficacy of MSC-based therapy for ischemic diseases by activating the Akt/mTOR/p70S6K pathway.

Keywords: Stem cell, apoptosis, oxidative stress, curcumin, Akt/mTOR/p70S6K pathway, hydrogen peroxide

Introduction

The therapeutic potential of MSCs has been proven in both preclinical and clinical studies, revealing a promising therapy for ischemic diseases and tissue damage [1, 2]. However, the low retention and poor survival of engrafted MSCs has hampered their therapeutic efficacy [3]. Oxidative stress-induced apoptosis is one of the primary reasons for the poor viability of engrafted MSCs [4]. The oxidative microenvironment, triggered by inflammation, nutrient and oxygen deprivation or loss of cellular adhesion to extracellular matrix (ECM), may induce pro-apoptotic signaling and cause cells/tissues damage. MSCs will suffer from an oxidative microenvironment that may induce their apoptotic death, once they were engrafted into ischemia-reperfusion damaged tissues or organs. Therefore, antioxidant treatments may be candidate strategies to improve the survival rates and therapeutic potential of transplanted MSCs.

Curcumin, a yellow pigmented substance and component of turmeric, has been proven to exert strong anti-oxidant, anti-inflammatory and anti-cancer properties [5]. The antioxidant capacity of curcumin is 100-fold stronger than that of vitamin E or C [6], and it has been used to function as an antioxidant and free-radical scavenger both *in vitro* and *in vivo* [5, 7]. The protective effect of curcumin against oxidative damage and its mechanisms have been report-

ed in various types of cells, including neuron cells [8], endothelial cells [9, 10], epithelial cells [11], and skin cells [12]. However, it is largely unknown whether and how curcumin affects the survival of MSCs exposed to oxidative stress.

A number of signaling pathways have evolved to protect cells against reactive oxygen species (ROS)-induced damage. The notable amongst them is the Akt/mTOR pathway which plays a vital role in promoting cell survival in response to oxidative stress [13, 14]. Although some studies demonstrated that curcumin inhibits Akt/mTOR pathway [15-17], this activity was seen principally in tumor cells and the effects may vary based on cell type; for example, curcumin induced apoptosis in human breast carcinoma and hepatoma cell lines, but failed to do so in normal rat hepatocyte primary cultures [18]. Furthermore, a recent study even demonstrated that H_2O_2 treatment caused a sustained inactivation of the PI3K/Akt pathway in PC12 cells, while pre-treatment with curcumin could significantly inhibit this inactivation [8]. Therefore, we hypothesized that curcumin could affect the survival of MSCs exposed to oxidative stress through influencing the Akt/mTOR/p70S6K pathway.

To test this hypothesis, we evaluated the survival of rat BM-MSCs exposed to H_2O_2 treatment with or without a curcumin pre-incubation procedure and detected the expression of key proteins in the Akt/mTOR/p70S6K pathway, and the role of this pathway in mediating the protective effects of curcumin was further confirmed by using the Akt inhibitor perifosine.

Materials and methods

Isolation and culture of BM-MSCs

All procedures in the present study were done in accordance with the "Guide for the Care and Use of Laboratory Animals, 8th Ed". Primary BM-MSCs were isolated from the long bones of rats by using the simple plastic-adherent method. Briefly, the tibias and femurs were removed, and then the bone marrow cells were harvested and cultured in UltraCULTURE™ Serum-free medium (LONZA; Walkersville, MD, USA) at 37°C in a humidified atmosphere of 5% CO_2. The non-adherent cells were removed 24 hours later, and the culture medium was replaced every other day. Adherent MSC clones were dissociated when they were visible to the naked eye, and the single-cell suspensions were then plated in new flasks and subcultured to 75%-85% confluence. In this study, BM-MSCs were used between the third and sixth passages.

Identification of BM-MSCs by flow cytometry (FCM) analysis

The third-passage BM-MSCs were harvested and suspended in PBS at a density of 2×10^6 cells/ml. The cell suspension was then stained with FITC-conjugated monoclonal antibodies against rat CD29, CD34, CD44 and CD45 (eBiosciences, San Diego, USA), as well as non-specific FITC-conjugated antibody, for 30 min at 4°C in the dark. After being washed twice with PBS, samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, USA).

Cell viability assay

Cell viability was assessed using the ATP assay. Briefly, BM-MSCs were plated into 96-well plates at a density of 1×10^4 cells/well. After 24 hours, they were treated with various concentrations of either H_2O_2 (0-800 μmol/L; Sigma, St Louis, MO, USA) or curcumin (0-100 μmol/L; Sigma) for indicated time period, or they were treated with 300 μmol/L H_2O_2 for 24 hours following a 3-hour pre-incubation with curcumin (0-100 μmol/L), respectively. After that, the culture medium was removed and the cells in each well were lysed by nuclear releasing buffer for 5 minutes at room temperature with gentle shaking. Next, the lysate was transferred into a standard opaque-wall 96-well plate for quantification of ATP by using a Luminescent ATP Detection Assay Kit (Jinzijing Bio-medicine Technology Co., Ltd., Beijing, China). The luminescence intensity was determined using a BHP9504 Luminometer (Hamamatsu, Japan). The ATP content was analyzed by running an internal standard, and was expressed as a percentage of untreated cells (control).

Cell proliferation assay

The xCELLigence Real-Time Cell Analysis (RTCA)-DP instrument (ACEA Biosciences, Hangzhou, China) was used to continuously monitor the proliferation and survival of BM-MSCs. First, 50 μL of complete culture medium was

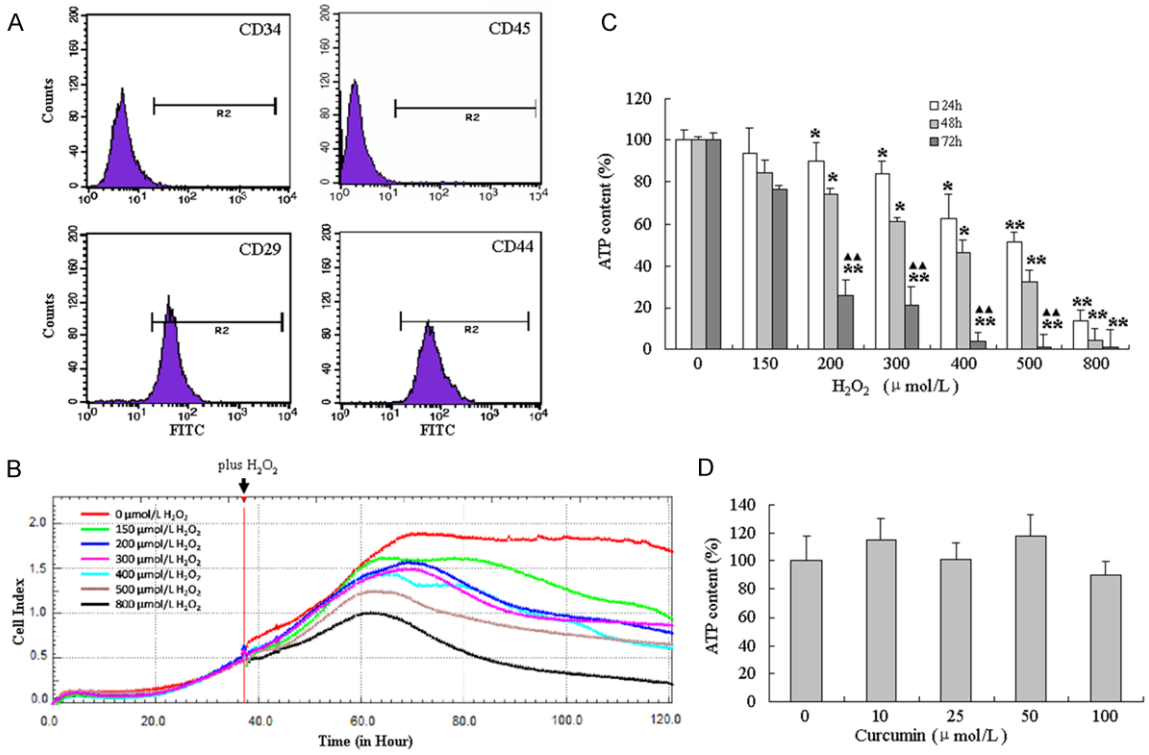

Figure 1. Immunophenotyped characteristics of BM-MSCs and cell viability of BM-MSCs assayed by ATP. A. Flow cytometry was used to determine the expression of surface markers on BM-MSCs. BM-MSCs expressed CD29 and CD44, but not CD34 and CD45. B. The cell proliferation was determined by xCELLigence Real-Time Cell Analysis (RTCA). C. BM-MSCs were incubated with the indicated concentrations of H_2O_2 (150-800 μM) for 24 h, 48 h and 72 h. Cell viability was then measured by ATP assay. H_2O_2 significantly decreased cell viability in BM-MSCs in a dose-dependent and time-dependent manner. D. Cell viability was determined by ATP assay after treatment of curcumin for 48 h. Each value represents the mean ± SD of four independent experiments. *P<0.05, **P<0.01, compared with the control group; ▲▲P<0.01, compared with the 48 h group.

added to each well of E-Plate 16 (ACEA Biosciences) for baseline inspection and quality control. Next, 150 μL of cell suspension was added and cell attachment was allowed (standing for 20 min at room temperature). The E-Plate 16 was then placed into the RTCA-DP device to noninvasively monitor the changes in electrical impedance at the bottom of the E-plate 16. Measured changes in electrical impedance were presented as cell index (CI) values, which evaluates cell proliferation for adherent cells. When BM-MSCs were in the logarithmic growth phase, the supernatant was dumped and 200 μL of culture medium containing different concentrations of H_2O_2 were added gently. Then, the CI values in each well were continuously monitored every 10 min.

Detection of cell apoptosis

Hoechst 33258 staining: Nuclear morphology changes in apoptotic BM-MSCs were investigated by labeling cells with the nuclear stain Hoechst 33258 (Sigma; St Louis, MO, USA). Briefly, BM-MSCs plated on the coverslips were treated with different concentrations of H_2O_2 for 24 h and then fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. Next, the cells were washed with PBS and stained with Hoechst 33258 (10 μg/ml) in darkness at room temperature for 10 min. After being washed three times with PBS, cells were observed by using a fluorescence microscopy (Olympus IX71, Japan).

Annexin V-FITC/PI staining followed by FCM analysis: After being treated with various concentrations of H_2O_2 for 24 h, both the adherent and suspended BM-MSCs were collected and double stained with Annexin V-FITC/PI (KeyGEN; Nanjing, China) in darkness at 4°C. Then, cell apoptosis was measured within 30 min by FCM. Both early (Annexin V-FITC$^+$PI$^-$) and late apoptotic (Annexin V-FITC$^+$PI$^+$) cells were included.

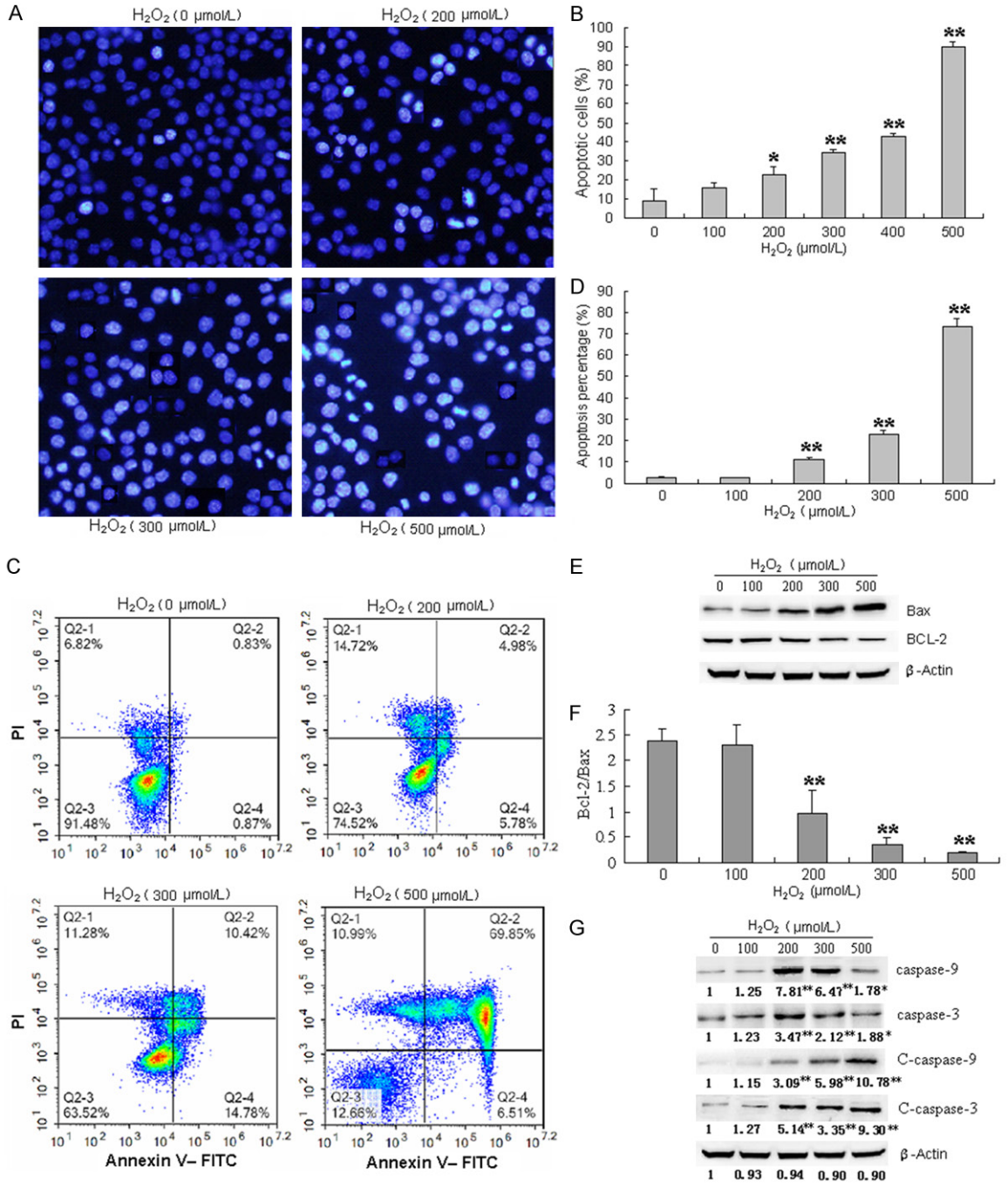

Figure 2. BM-MSCs apoptosis is induced by H_2O_2. BM-MSCs were treated with the indicated concentrations of H_2O_2 for 24 h. A. Nuclear staining with Hoechst 33258 was performed to examine the rate of apoptosis of BM-MSCs. Scale bar =20 μm. B. Cell apoptosis was evaluated by Hoechst 33258 staining. C, D. The apoptotic percentage of BM-MSCs was analyzed by flow cytometry. Each value represents the mean ± SD of five independent experiments. E, F. Expressions of Bcl-2 and Bax were assessed by western blot analysis. G. Expressions of total and cleaved caspase-3 and caspase-9 assessed by western blot analysis. Each value represents the mean ± SD of three independent experiments. *P<0.05, **P<0.01, compared with the control group.

Western blot analysis

BM-MSCs in the logarithmic growth phase were treated with various concentrations of H_2O_2 (0-500 μmol/L) for 24 h. For another set of experiments, either a 3-hour pre-incubation with curcumin or a 2-hour pre-incubation with Akt inhibitor perifosine (Selleckchem; Houston,

TX, USA), or a combination of both procedures was adopted, followed by treatment with H_2O_2 (300 μmol/L) for 24 h. Cells were then collected and lysed in RIPA lysis buffer (Beyotime, Haimen, China) to extract the total proteins, and the protein concentrations were determined using a BCA Protein Assay Kit (Biosharp, Hefei, China). Equal volumes of proteins (80 μg) from each sample were separated by SDS-PAGE (8%-12% gels) and transferred to PVDF membranes. Then, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline for 60 min at room temperature and incubated with primary antibodies (anti-Akt, anti-p-Akt (Thr308), anti-mTOR, anti-p-mTOR (Ser-2481), anti-p70S6K, anti-p-p70S6K (Thr421/Ser424), anti-Bcl-2, anti-Bax, anti-cleaved caspase 9, and anti-cleaved-caspase 3; CST, Beverly, MA, USA) overnight at 4°C. After incubation with the corresponding HRP-conjugated secondary antibodies (Zhongshan Goldenbridge Bio-Tech Co., Beijing, China), the membranes were detected by enhanced chemiluminescence (GE Healthcare, USA). Band density was analyzed using LabWorks 4.6 analysis software.

Statistical analysis

All statistical analyses were performed using SPSS13.0 software (SPSS Inc., USA). Data were expressed as mean ± standard deviation (SD). Differences between multiple groups were analyzed by analysis of variance (ANOVA), while differences between two groups were analyzed using a *t* test. A p value <0.05 was considered statistically significant.

Results

Effect of H_2O_2 and curcumin on the cell viability of BM-MSCs

As shown in **Figure 1A**, the BM-MSCs used in this study were positive for CD29 and CD44 and negative for CD34 and CD45, which confirms to the concept of MSC [19]. The continuous RTCA results showed that the proliferation and viability of BM-MSCs were inhibited gradually with the increase of H_2O_2 concentration over the tested concentration range (150-800 μmol/L) (**Figure 1B**). By using the ATP assay, we further confirmed that H_2O_2 decreased the overall viability of BM-MSCs in a concentration- and time-dependent manner (**Figure 1C**), while curcumin (0-100 μmol/L) has no obvious effect on BM-MSC viability (**Figure 1D**).

H_2O_2 induces apoptosis of BM-MSCs via mitochondrial pathways

We inferred that the H_2O_2-induced decrease in overall cell viability of BM-MSCs was attributed to cell apoptosis. As predicted, 24-hour treatment with H_2O_2 induced significant apoptosis in BM-MSCs, as shown by Hochest 33258 staining (**Figure 2A**, **2B**) and FCM analysis (**Figure 2C**, **2D**). The balance of Bcl-2/Bax inhibits cell apoptosis by maintaining mitochondrial membrane stability and preventing mitochondrial membrane depolarization and cytochrome c release into the cytoplasm [20]. As shown in **Figure 2E**, **2F**, H_2O_2 induced an obvious decrease in Bcl-2/Bax ratios, in a dose-dependent manner. Thus, we speculated that H_2O_2 induced BM-MSC apoptosis via mitochondrial pathway. As caspase-9 and caspase-3 are two key mediators of mitochondrial cell death [21], we then examined the expression levels of caspase-9/3 and cleaved caspase-9/3 in H_2O_2-treated BM-MSCs. The results showed that H_2O_2 induced an activation in both caspase-9 and caspase-3 in a dose-dependent manner (**Figure 2G**), confirming our speculation.

Curcumin protects BM-MSCs against H_2O_2-induced apoptosis

To test the potency of curcumin in protecting MSCs against oxidative stress-induced damage, we pre-incubated BM-MSCs with curcumin before they were exposed to H_2O_2. As shown in **Figure 3A**, a 3-hour pre-incubation with curcumin could largely improve the viability of BM-MSCs exposed to H_2O_2 (300 μmol/L) in a dose-dependent manner, and this protective effect of curcumin reached the maximum at 25 μmol/L. The FCM analyses further confirmed that pre-incubation with curcumin (6.25-25 μmol/L) could significantly inhibited apoptosis of BM-MSCs challenged by 300 μmol/L H_2O_2 for 24 hours (**Figure 3B**, **3C**). Therefore, in the following experiments, the curcumin concentration was fixed at 25 μmol/L. We found that pre-incubation with curcumin could significantly improve the Bcl-2/Bax ratio and inhibit the activation of caspase-9 and caspase-3 in H_2O_2-challenged BM-MSCs (**Figure 3D**).

Curcumin exerts its protective effects through inhibiting H_2O_2-induced inactivation of Akt/mTOR/P70S6K pathway

Next, we attempted to explore the underlying molecular mechanism of inhibition of H_2O_2-

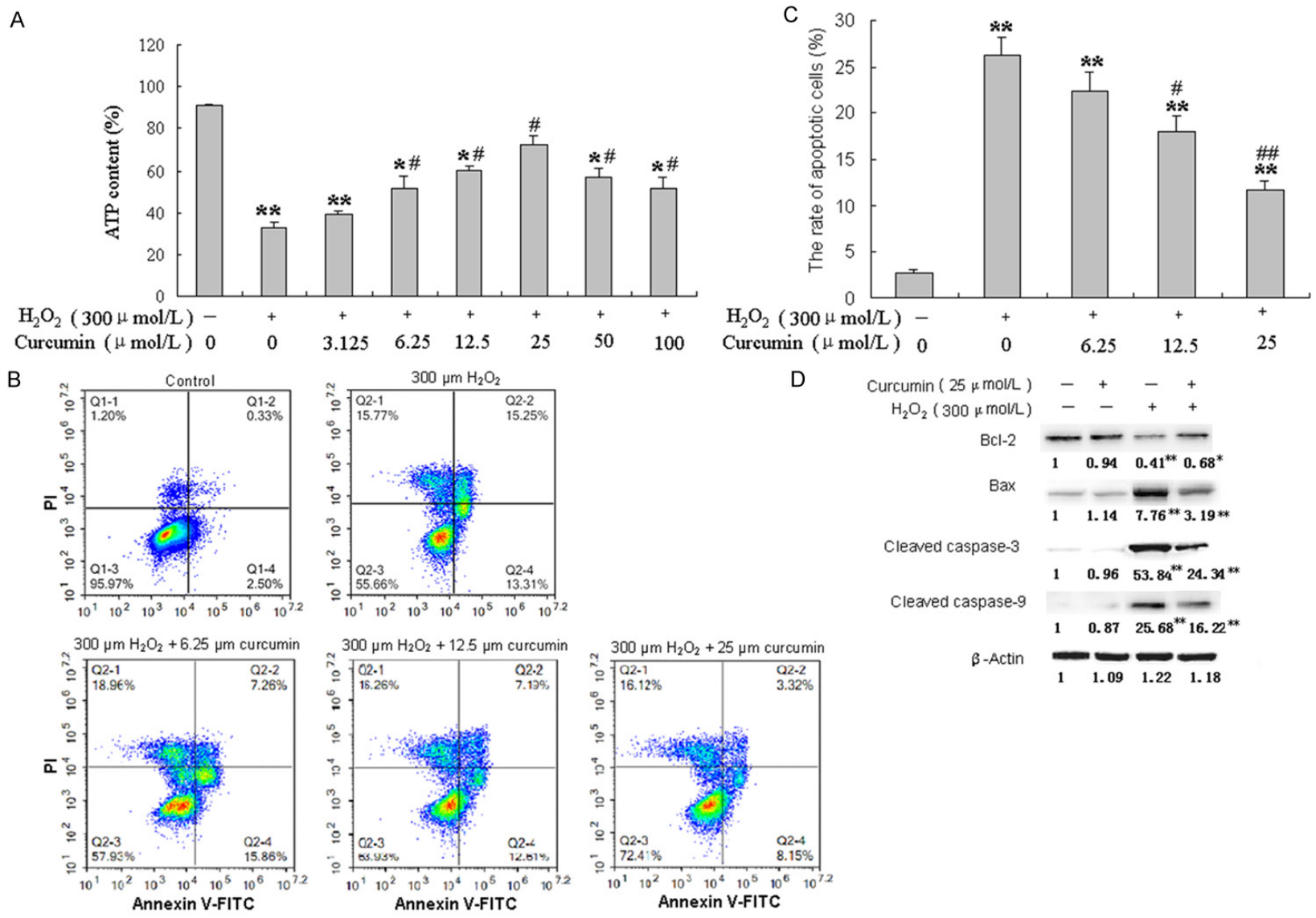

Figure 3. Cytoprotective effects of curcumin on H_2O_2-induced apoptosis in BM-MSCs. A. BM-MSCs were treated with 0-100 μM curcumin in the presence or absence of 300 μM H_2O_2 for 24 h. The cell viability was measured by ATP assay. Each value represents the mean ± SD of four independent experiments. B, C. BM-MSCs were pre-treated with the indicated concentrations of curcumin for 3 h before exposure to 300 μmol/L H_2O_2 for 24 h. The rate of apoptotic cells was assayed by flow cytometry. Each value represents the mean ± SD of three independent experiments. D. Expression of Bcl-2, Bax, cleaved caspase-9 and caspase-3 was assessed by western blot analysis. *P<0.05, **P<0.01, compared with the control group (curcumin 0 μmol/L, H_2O_2 0 μmol/L); #P<0.05, ##P<0.01, compared with H_2O_2 300 μmol/L group.

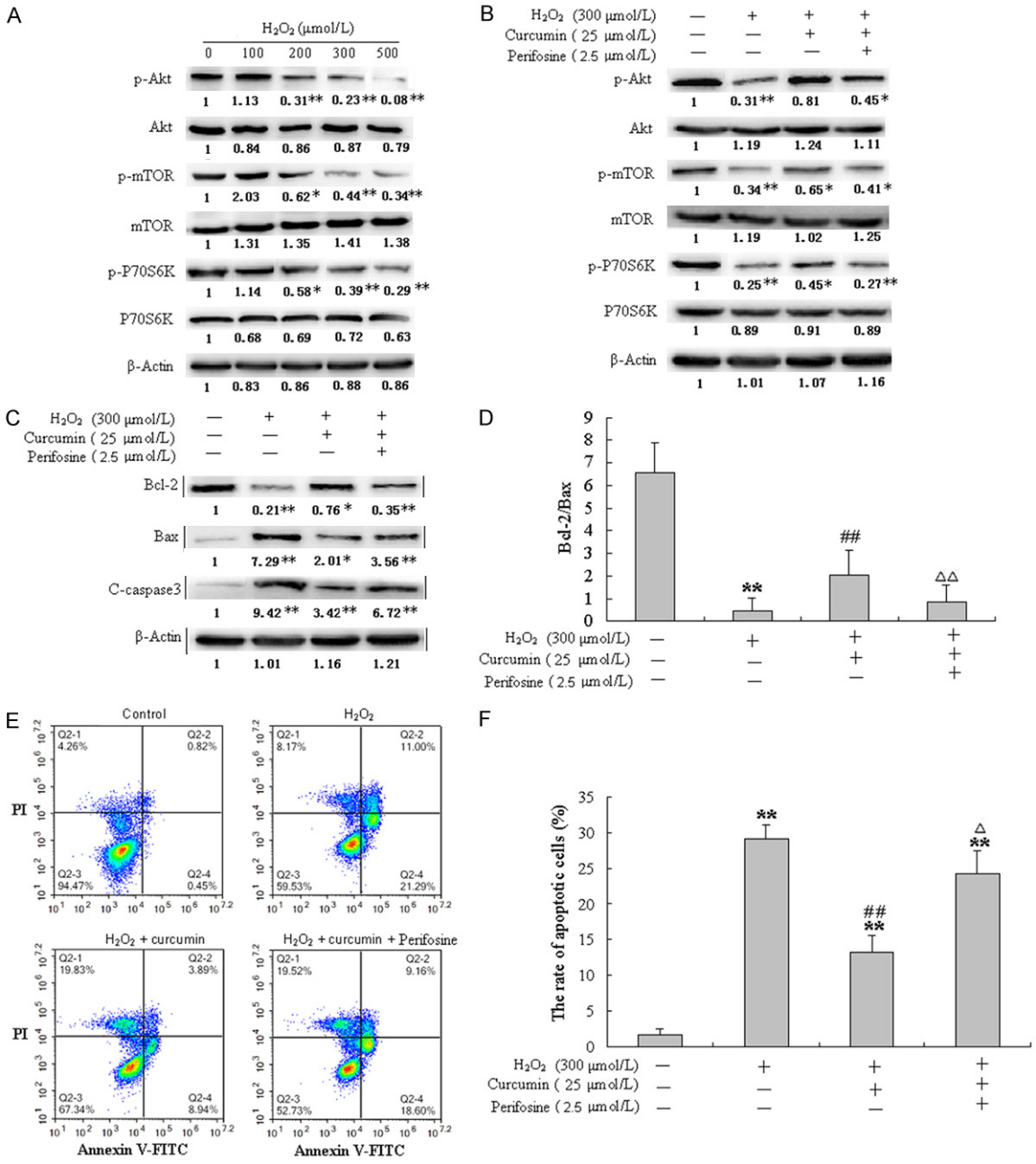

Figure 4. The protective effect of curcumin is mediated by the activation of Akt/mTOR/p70S6k signaling. A. After 24 h treatment with different concentrations of H_2O_2, Western blot analysis was performed to examine total and phosphorylation of Akt, mTOR and p70S6K. B. After 2 h pre-incubation with 50 ng/ml rapamycin, BM-MSCs were treated with 300 μmol/L H_2O_2 in the absence or presence of curcumin for 24 h. Western blot analysis was performed to examine total and phosphorylation of Akt, mTOR and p70S6K. C. Western blot analysis was performed to examine the expression of Bcl-2, Bax and cleaved-caspase 3. D. The rate of Bcl-2/Bax. E, F. The rate of apoptotic cells was assayed by flow cytometry. Each value represents the mean ± SD of three independent experiments. *P<0.05, **P<0.01, compared with the control group (curcumin 0 μmol/L, H_2O_2 0 μmol/L); $^{\#}$P<0.05, $^{\#\#}$P<0.01, compared with H_2O_2 300 μmol/L group. $^{\Delta}$P<0.05, compared with the H_2O_2 plus curcumin group.

induced apoptosis in BM-MSCs by curcumin. Akt/mTOR pathway plays a vital role in promoting cell survival in response to oxidative stress [13, 14], while curcumin can influence this pathway [8, 17], we thus focused our exploration on Akt/mTOR/p70S6K pathway. As shown

in **Figure 4A**, although there was no obvious alteration in the total levels of Akt, mTOR and p70S6K in BM-MSCs after being treated with various concentrations of H_2O_2 for 24 hours, the phosphorylation levels of these proteins increased with increasing H_2O_2 concentration, indicating a H_2O_2-induced inactivation of the Akt/mTOR/p70S6K pathway in BM-MSCs. We then pre-incubated BM-MSCs with curcumin alone or together with perifosine to evaluate whether curcumin exert its protective effects against oxidative damage through affecting the Akt/mTOR/p70S6K pathway. The results showed that a pre-incubation with curcumin could largely prevent the H_2O_2-induced inactivation of Akt/mTOR/p70S6K pathway, while a simultaneous pre-incubation with perifosine significantly counteracted the effects of curcumin (**Figure 4B**). Similarly, we found that pre-incubation with curcumin largely inhibited the H_2O_2-induced elevation of cleaved-caspase-3 and Bax/Bcl-2 ratio, while a simultaneous pre-incubation with perifosine significantly reversed the effects of curcumin (**Figure 4C**, **4D**). By using FCM analysis, we further confirmed that the protective effect of curcumin against H_2O_2-induced BM-MSC apoptosis was obviously counteracted by perifosine (**Figure 4E**, **4F**). We concluded from these results that the protective effect of curcumin against oxidative stress-induced BM-MSC apoptosis was mediated, at least in part, through inhibiting the inactivation of Akt/mTOR/p70S6K pathway.

Discussion

MSCs have been proposed to be a promising cell population for use in cell therapy and regenerative medicine [22]. However, the efficacy of MSC transplantation therapy in ischemic disease is limited due to the poor survival of implanted cells, and oxidative stress may be a major reason for the poor survival of transplanted cells [23]. Curcumin is the principal curcuminoid of turmeric with strong antioxidant activity, which has been reported to inhibit H_2O_2-induced oxidative damage in several somatic cell types [8-12]. A recent study has reported the protective effects of curcumin against H_2O_2-induced cell death in adipose-derived MSCs [24], but it needs more evidence and the mechanisms need more investigation. In the present study, we also found a pro-apoptotic effect of H_2O_2 and a protective effect of curcumin against H_2O_2-induced apoptosis in bone marrow-derived MSCs, and we further confirmed the pivotal role of Akt/mTOR/p70S6K signaling pathway in mediating the pro-apoptotic effect of H_2O_2 and the protective effect of curcumin.

H_2O_2, the most stable compound among ROS and the most feasible molecule for ROS-mediated signal transduction, is often used to establish oxidative stress injury models *in vitro* to study apoptosis and anti-apoptosis mechanisms [25]. Our results showed that H_2O_2 strongly induced the apoptosis of BM-MSCs in dose-dependent manner, suggesting the successful nature of our acute oxidative stress injury model. Sustaining the equilibrium between the anti-apoptotic Bcl-2 and pro-apoptotic Bax is essential for regulating mitochondrial integrity and preventing cytochrome c release into the cytoplasm. Cytochrome c released from mitochondria will bind procaspase-9 and Apaf-1, as well as dATP to cleave and activate caspase-9. Activated caspase-9 directly cleaves and activates pro-caspase-3, initiating a cascade of additional caspase activation that culminates in cell apoptosis [26]. Here, we found an up-regulation in expression of Bax, cleaved-caspase-9 and -3 and a down-regulation in expression of Bcl-2 in H_2O_2-treated BM-MSCs, suggesting that H_2O_2 induces BM-MSC apoptosis through the mitochondrial pathway, which shines light on exploring new drugs capable of rescuing BM-MSCs from ROS-induced apoptosis.

Curcumin has been shown to be a potent scavenger of ROS and to inhibit lipid peroxidation *in vitro* and *in vivo* [6, 7]. In this study, we presented new direct evidence demonstrating the cytoprotective effects of curcumin against H_2O_2-induced apoptosis in BM-MSCs, which were accompanied by increased expression of Bcl-2 and decreased expression of Bax, cleaved-caspase-9 and -3, suggesting that curcumin exerts its anti-apoptotic effect through preventing the mitochondrial apoptosis pathway mediated by the disequilibrium between the Bcl-2 and Bax.

The Akt/mTOR/p70S6k pathway has been shown to play a major role in the promotion of cell survival and prevention of apoptosis in response to oxidative stress [8, 14, 27, 28]. Akt promotes cell survival and protects against

apoptosis by both enhancing the expression of anti-apoptotic proteins Bcl-2 and inhibiting the activity of pro-apoptotic proteins Bad, Bax and caspase-9 [29, 30]. We observed an obvious decrease in phosphorylated Akt, mTOR and p70S6K, indicating an inactivation of Akt/mTOR/p70S6k pathway, in BM-MSCs after they were treated with H_2O_2. The H_2O_2-induced inactivation of Akt/mTOR/p70S6k pathway in BM-MSCs was largely eliminated by a pre-incubation with curcumin, and the specific Akt inhibitor perifosine largely reversed the effects of curcumin. Furthermore, the H_2O_2-induced mitochondrial apoptosis of BM-MSCs was closely bound up with the inactivation of Akt/mTOR/p70S6k pathway. Therefore, we conclude that the restoration of the activity of Akt/mTOR/p70S6k signaling pathway is necessary for curcumin's pro-survival functions. However, it should be noted that there may be other signaling pathways mediating the protective effects of curcumin against H_2O_2-induced apoptosis, which cannot be entirely excluded based on the current results. Moreover, it would be more integrated if the expression of the upstream enzymes, such as PI3K and ERK1/2, that are associated with the phosphorylation of Akt, mTOR, and p70S6k have been investigated.

In summary, we demonstrated in this study that curcumin can effectively inhibit H_2O_2-induced inactivation of the Akt/mTOR/p70S6K pathway and cell apoptosis in BM-MSCs, and the Akt inhibitor perifosine can largely abolish the protective effects of curcumin, which suggests that curcumin protects BM-MSCs against oxidative stress-induced damage through inhibiting the inactivation of Akt/mTOR/p70S6K pathway. We believe that pre-incubating BM-MSCs with curcumin before they were transplanted into oxidative environment represents a novel and promising strategy to improve the survival and therapeutic efficacy of transplanted cells.

Acknowledgements

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Disclosure of conflict of interest

None.

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