NMNAT3 improves mitochondrial function and enhances BMSCs anti-oxidative stress through the NAD+-Sirt3 pathway

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Research

Keywords: oxidative stress, nicotinamide adenine dinucleotide, nicotinamide mononucleotide adenylyl transferase 3, mitochondrial function, bone marrow mesenchymal stem cells

Posted Date: March 13th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-17046/v1

Abstract

Background To investigate the effects of NMNAT3 on mitochondrial function and anti-oxidative stress in rabbit BMSCs and its underlying mechanisms.

Methods Stable strains of NMNAT3 overexpressing rabbit BMSCs were obtained by lentivirus transfection; the Oxidative stress model in rabbit BMSCs was imitated by treating with H 2 O 2 ; Observe the changes in mitochondrial ultrastructure and mitochondrial function-related indicators (mitochondrial membrane potential, ATP and mitochondrial protein PGC-1α, NRF1 synthesis), to study the effect of NMNAT3 on improving mitochondrial function under oxidative stress; detect ROS and lipids Peroxidation products (MDA content), antioxidant enzymes (CAT and GPx) activity, SA-β-Gal activity and apoptosis in rabbit BMSCs, to study the changes of the anti-oxidative stress ability of BMSCs modified by NMNAT3;Then, the selective Sirt3 inhibitor (3-TYP) was used to analyze the mechanism of NMNAT3 improve mitochondrial function and antioxidant stress ability of BMSCs by regulating mitochondrial NAD + levels, and whether it affects the acetylation levels of mitochondrial target proteins (Idh2 and FOXO3a) and the expression and activation of effect proteins (GSH, ATP synthase and Mn-SOD) through Sirt3 pathway.

Results Overexpression of NMNAT3 can improve the mitochondrial ultrastructural damage of rabbit BMSCs under oxidative stress; increase the mitochondrial membrane potential, increase the synthesis of ATP and mitochondrial protein PGC-1α and NRF1,significantly improve the function of mitochondria.It also can significantly enhance the antioxidant stress ability of rabbit BMSCs by reducing ROS level and MDA content, increasing the activities of antioxidant enzymes CAT and GPx, and decreasing cell senescence and apoptosis under stress.Rabbit BMSCs overexpressed by NMNAT3 significantly increased the activity of Sirt3, significantly decreased the acetylation levels of Idh2 and FOXO3a under oxidative stress, and increased the level of GSH and the activities of ATP synthase and Mn-SOD.However, the addition of 3-TYP basically blocked the protective effect of NMNAT3, indicating that Sirt3 is an important pathway for NMNAT3 to regulate mitochondrial function and antioxidant stress in BMSCs under oxidative stress.

Conclusion NMNAT3 can effectively improve the mitochondrial function of rabbit BMSCs under oxidative stress through NAD + -Sirt3 pathway, and play a role in enhancing its antioxidant stress injury.

Background

The survival of stem cell transplantation is the key problem to be solved in current research and clinical treatment[1–3]. Studies have shown that oxidative stress damage is often caused by the microenvironment such as ischemia, hypoxia and inflammation in the transplantation area, which seriously affects the mitochondrial function of stem cells and limits the proliferation, differentiation and survival of implanted stem cells. it accelerates the aging and apoptosis of transplanted stem cells, which leads to poor curative effect in vivo[4–7]. Therefore, improving the mitochondrial function of stem cells in

the transplantation area and enhancing its anti-stress ability may be an effective way to improve the survival rate and curative effect of stem cell transplantation[8–11].

In recent years, research on the molecular mechanism of mitochondrial disorders have shown that nicotinamide adenine dinucleotide (NAD^+) is a key regulator in mitochondria, and the level of NAD^+ directly controls important life processes such as cell rhythm, senescence, resistance and cytoprotection [12–14], which is mainly related to various enzymes dependent on NAD^+. For example, involved in stress and aging-related protein deacetylation reactions, polyadenylate diphosphate ribose reactions that play important roles in DNA repair, and adenosine diphosphate ribose cyclization reactions that regulate calcium channels and so on[15–18]. Among them, the deacetylation modification of mitochondrial proteins plays an important role in regulating the functional stability of mitochondria and the adaptation to biological and chemical stressors[19,20], which is mainly mediated by the silent mating type information regulation 2 homolog 3 (Sirt3) of the sirtuins gene family[21–24]. The deacetylase activity of Sirt3 depends on NAD^+ activation. When the content of NAD^+ in mitochondria is depleted, it can lead to the decrease of Sirt3 activity, which directly affects the stability of energy metabolism and the balance of antioxidant defense system in mitochondria[25–28]. Therefore, interfering with the regulation of NAD^+ homeostasis in mitochondria and then improving the activity of Sirt3 is an important means to improve mitochondrial dysfunction[29].

Among many enzymes involved in the regulation of NAD^+ homeostasis, nicotinamide mononucleotide adenylyl transferase 3 (NMNAT3) has been found to be essential for the formation of NAD^+ in mitochondria[30–32]. Overexpression of NMNAT3 can significantly increase the levels of NAD^+ in mitochondria of aged mouse tail-tip fibroblasts and human umbilical cord blood mesenchymal stem cells, restore Sirt3 activity, and delay cell aging and apoptosis[33]. Similarly, Gulshan et al. have further demonstrated that NMNAT3 can be used as a target enzyme to increase the level of mitochondrial NAD^+ in NMNAT3 transgenic mice, which is important to improve mitochondrial biosynthesis and energy metabolism, reduce the level of reactive oxygen species (ROS) and restore mitochondrial function[34]. However, at present, the effect of NMNAT3 on the mitochondrial function and anti-stress ability of stem cells in the transplantation area has not been studied, and it is unclear whether it will help improve the survival rate and efficacy of stem cell transplantation.

In this study, we overexpressed the NMNAT3 gene in bone marrow mesenchymal stem cells (BMSCs) in vitro. We simulated the microenvironment of oxidative stress in the transplantation area by H_2O_2 to evaluate the effects of NMNAT3 on the mitochondrial function and antioxidant stress ability of BMSCs under oxidative stress[35]. We also hope to further clarify the possible regulatory mechanism of NMNAT3, provide a new method to solve the problem of survival of stem cell transplantation, promote the clinical transformation of stem cells,and open up more new ideas for treatment.

Materials And Methods

Animals

all the experimental animals used in this study were provided by the Animal Experimental Center of Guizhou Medical University: A total of 20 young New Zealand white rabbits (2.0 ± 0.5 kg). All animals are approved by the Experimental Animal Bioethics Committee of Guizhou Medical University (Grant No.1900590), and all procedures are carried out in strict accordance with the guidelines for the Care and use of Experimental Animals issued by the National Institutes of Health (NIH publication No. 85-23, 1996 revised).

Cell culture

The rabbits were fixed on the operating table of small animals in the supine position, and routinely disinfected and laid towels. The distal femur and proximal tibia of rabbits were anesthetized with 2% lidocaine hydrochloride (ShandongHualuPharmaceuticalCo.,Ltd.,China), and then punctured. The bone marrow solution was extracted from 4-5 mL, and then the cells were separated by density gradient centrifugation[36,37]. The complete L-DMEM (Gibco,USA) medium containing 10%FBS (Gibco,USA) and 1% double antibody (Hyclon,USA) was inoculated in the 25cm^2 culture bottle (Corning,USA) and cultured at 37 ℃ in the 5%CO_2 incubator (Thermo,USA). After that, the complete medium was changed every 3 days, and when the confluence of the cells at the bottom of the culture bottle reached more than 90%, the BMSCs began to be digested and passaged at 1:3.

Mitochondrial extraction

The treated BMSCs was washed with PBS, the cells were digested by trypsin, and the cells were collected and counted. 5×10^7 cells were extracted and added to 1.0mL ice-precooled PBS resuspended cells. The cell suspension was transferred into a small volume glass homogenizer and grinded in an ice bath at 0 ℃ for 30 times to obtain cell homogenate. Isolation and extraction of mitochondria according to the steps of the cell mitochondria separation kit (Beytime,China), and the mitochondrial precipitation was resuscitated with 50-100 μL Store Buffer or suitable reaction buffer, and then used immediately or stored at -70 ℃.

BMSCs surface antigen identification

The third generation of rabbit BMSCs, was digested and centrifuged to adjust the cell concentration to make 2.0×10^7 cells/mL single cell suspension. 50μL cell suspension was taken into the flow tube (Corning,USA) and added with Anti-CD29/AF647, Anti-CD90/PE-Cy^{TM}7, Anti-CD106/PE, Anti-CD45/FITC and Anti-CD11b/V450 (BD,USA), respectively. Buffer (Hyclon,USA) was used as negative control, and the

supernatant was centrifuged to remove the supernatant after incubation at room temperature for 30 min, 1000 rpm/min and 5 min. Each tube was washed with buffer twice, and then 500uLbuffer resuspension cells were added to each tube and detected by flow cytometry (Beckman,USA).

BMSCs multidirectional differentiation induction

The third generation of rabbit BMSCs, with good growth was selected and the cell concentration was adjusted to 2.0×10^4 cells/cm^2, according to osteogenic, adipogenic and chondrogenic induction differentiation kit (CyagenBiosciencesSuzhouInc.,China). The cells were inoculated on a 6-well plate with 2ml per well. When the cell convergence degree reached 60%,100% and 60% respectively, the experimental group changed the induced differentiation medium respectively, while the control group continued to use complete L-DMEM medium. The operation is carried out according to the instructions of each induction kit. After the induction is completed, the culture medium is absorbed, washed twice with PBS (Hyclon,USA), 4% paraformaldehyde (BeijingLeageneBiotech.Co.,Ltd.,China) of 1mL is added to each well, fixed at room temperature for 30 minutes, and each well is washed twice with PBS buffer, according to the staining instructions of each kit, Alizarin red staining solution, oil red O staining solution and alisin blue staining solution were used for staining (CyagenBiosciencesSuzhouInc.,China), and observed and photographed under inverted microscope.

Lentivirus infection

The third generation of rabbit BMSCs, were divided into three groups according to the transfection conditions: group A (BMSCs), group B (BMSCs+Lv-EGFP) and group C (BMSCs+Lv-NMNAT3-EGFP). Lentivirus transfection (ShanghaiHengYuanBiologicalTechnologyCo.,Ltd.,China) (MOI=100) was performed for 12 hours according to the experimental group[37]. After 3 days, the expression of green fluorescent protein was observed by inverted fluorescence microscope and the transfection efficiency was calculated. After 5 days of transfection, the culture medium containing puromycin (Solarbio,Beijing,China) (2ug/mL) was added for screening. After all the cells in the control group died, the concentration of puromycin was halved (1ug/mL) to obtain stable strains.

Real-time RT-PCR

Isolation and extraction of total RNA from cells using Trizol(Invitrogen,USA),take 10 μL of total RNA for reverse transcription using RevertAid™ 1st-strand cDNA Synthesis Kit (SangonBiotech,China). The reaction parameters were as follows: 65 ℃, 5 min; 42 ℃, 30 min; 70 ℃, 10 min. 1 μL reverse transcriptional product cDNA was used for real-time fluorescence quantitative PCR, cycle parameters: 95 ℃, 3 min; 95 ℃, 3 min; 60 ℃, 30 s; 40 cycles. At the same time of determining the target gene, the

endogenous butler gene (β-actin) , was determined with the internal reference as the comparative standard. Using the comparative threshold method: the relative expression quantity of the target gene $=2^{-\triangle\triangle Ct}$, was converted into the relative quantitative relationship of the initial template number of the sample.Rabbit primers (Sangon Biotech) were as follows: NMNAT3-F: CCCGTCAATGACAGCTACAGGAAG; NMNAT3-R: AGCACCTTCACCGTCTCCATCC; β-actin-F: TCCCTGGAGAAGAGCTACGA; β-actin: GTACAGGT CCTTGCGGATGT.

Immunoblot and Immunoprecipitation

The total protein of each group was extracted by cell lysis, and the protein concentration of each group was determined by BCA method. After adjusting the protein concentration, the protein was boiled and denatured, and then electrophoretic, membrane transfer and sealing were carried out in turn. Than the target first antibody (Anti-NMNAT3、Anti-PGC-1α、Anti-NRF1、Anti-Sirt3、Anti-IDH2、Anti-FOXO3a) (diluted by 1: 300) and internal reference antibody (Anti-β-actin、Anti-COX IV) (diluted by 1: 7500) were added respectively and incubated overnight at 4 ℃. The TBST film was washed for 3 times, then incubated at room temperature with secondary antibody (diluted by 1: 7500) for 1h, and then washed for 3 times. Finally, the film was exposed by Fusion Fx exposure machine. β-actin and COX IV were used as an internal reference protein, and the relative expression of the target protein was calculated according to the gray value of protein band/β-actin(or COX IV) protein band gray value. For co-immunoprecipitation, the cell protein was also extracted by lysate and quantified by BCA method, then the protein was added to ProteinA/G agarose beads and incubated at 4 ℃ for 2 hours, and the supernatant was obtained by 2500rpm centrifugation for 3min at 4 ℃, and the corresponding antibody was put in the refrigerator at 4 ℃ and slowly rotated to incubate overnight. The next day, ProteinA/G agarose beads were slowly incubated at 4 ℃ for 3h, 2500rpm centrifugation for 3 minutes, and the precipitate was slowly washed for 3 times. For the last time, carefully absorb the supernatant and add the sample buffer to the precipitation to mix well; boil at 95 ℃ for 5min and centrifuge at 12000rpm for 1min; Western Blot detection was carried out according to the above method.

Cellular oxidative stress

After the stable strain of BMSCs overexpressing NMNAT3 gene was obtained, according to the experimental group, each group was treated with H_2O_2 with a concentration of 600μM BMSCs for 24 hours[35], and the control group continued to be cultured in complete L-DMEM medium.

Observation of mitochondrial ultrastructure with electron microscope

After the BMSCs treatment of each group, routine digestion and centrifugation, EP tube to collect cells, add 3% glutaraldehyde to fix, then dehydration, osmosis, entrapment and other treatments, and make ultra-thin sections, electron staining, transmission electron microscopy to observe the ultrastructural changes of mitochondria, and select a typical visual field to take pictures.

JC-1 staining

According to the instructions of JC-1 mitochondrial membrane potential detection kit, the treated cells were incubated with dye mixture at 37 ℃ for 30 minutes and washed gently for 3 times. the cells were observed under laser confocal microscope and recorded by random typical visual field. The red / green fluorescence ratio was calculated by Image software.

NAD^+ content detection

According to the experimental group, the well-growing BMSCs was taken for routine digestion, the cells were suspended in the complete culture medium, the appropriate cell concentration was adjusted, and the cells were inoculated in 96-well plate to continue culture. after the cell growth density met the requirements, the operation was carried out according to the instructions of NAD^+ detection kit. After the cells of each group were treated, the standard curve was drawn by enzyme labeling instrument, and the absorbance at 520nm wavelength of each group was recorded and calculated.

ATP levels detection

According to the experimental group, the well-growing BMSCs was taken for routine digestion, the cells were suspended in the complete culture medium, the appropriate cell concentration was adjusted, and the cells were inoculated in 96-well plate to continue culture. after the cell growth density met the requirements, the operation was carried out according to the instructions of ATP detection kit. After the cells of each group were treated, the standard curve was drawn by enzyme labeling instrument, and the absorbance at 532nm wavelength of each group was recorded and calculated.

DCFH-DA staining

After the third generation of BMSCs was digested and centrifuged, the cell density was adjusted to 2.5×10^4/ mL,1mL/ dish and cultured in confocal petri dish, and then treated according to the conditions of each group. Finally, the cells in each group were treated strictly in accordance with the instructions of

the ROS detection kit, the red fluorescence channel was detected under confocal microscope, and the pictures were collected.

MDA content detection

The third generation of BMSCs was digested and centrifuged and inoculated in 96-well plates with 2.5×10^3 cells per well. Then, according to the treatment conditions of each group, the cells were lysed with IP cell lysate, and the cell lysate was collected and centrifuged in EP tube to absorb the supernatant. Finally, strictly according to the instructions of the (MDA) determination kit, the absorbance value was measured and calculated at the 532nm wavelength by the enzyme labeling instrument.

β-gal staining

According to the experimental group, the cells were fixed at room temperature for 15 minutes, and the staining solution was prepared according to the cell senescence β-galactosidase staining kit. According to the instructions of the kit, after adding the working solution, the cells were cultured overnight at 37 ℃ and observed with an ordinary optical microscope.

Cell viability and proliferation

After routine trypsin digestion of BMSCs in each group, the complete culture medium was suspended and inoculated into 96-well plates according to (2.0-3.0) × 10^3/well. There were 4 multiple holes in each group, a total of 8 plates, and continued culture in 37 ℃ and 5%CO_2 incubator. After that, one plate was taken at the fixed time every day according to the operation instructions of CCK-8 kit. After the cells of each group were treated, the absorbance (OD value) of each group at 450nm was measured by enzyme labeling instrument, and continuously monitored for 8 days. The cell growth curve of each group was drawn with the cell culture days as the horizontal axis and the average absorbance value of each group as the vertical axis.

TUNNEL/DAPI detection of apoptosis

According to the experimental group, the treated cells were digested and centrifuged, and the cell density was adjusted to 2.5×10^4 / mL,1mL/ dish, then inoculated in confocal petri dish, and then treated according to each treatment condition. Finally, the cells of each group were treated strictly in accordance with the instructions of TUNEL and DAPI detection kit, observed under confocal microscope, and the pictures were collected.

Statistical analysis

All statistical data were calculated and graphed using GraphPad Prism software version 6 (GraphPad Software, San Diego, California, USA). All numerical data are expressed as mean ± standard deviation (SD) (±SD). Statistical significance was evaluated using paired two-tailed Student's t-test. Differences were considered significant at $p<0.05$.

Results

Overexpression of NMNAT3 in BMSCs and increased of Mitochondrial NAD^+ level

We used NMNAT3-loaded lentivirus to transfect BMSCs and selected stable strains. The mRNA expression of NMNAT3 was detected by qRT-PCR and the protein expression of NMNAT3 was evaluated by Western Blot. The results of qPCR showed that the level of mRNA in overexpression group was significantly higher than that in control group and the empty virus group ($^*P < 0.05$). The expression was up-regulated by (3.475±0.263) times, and there was no significant difference between the control group and the empty virus group (Fig. 1A). Western Blot results showed that positive protein bands with molecular weight of about 28kDa were detected in each group of BMSCs (Fig. 1B), and the expression of NMNAT3 protein in overexpression group was significantly higher than that in control group or empty virus group, and the protein content was increased approximately by (2.844±0.152) times (Fig. 1C). These results confirmed the successful overexpression of NMNAT3 gene in BMSCs.

NMNAT3 is a key enzyme in the synthesis of NAD^+ in mitochondria and is essential for the steady-state regulation of NAD^+ in the mitochondria[30-34]. In order to further observe the effect of NMNAT3 overexpression on the levels of NAD^+ in mitochondria, according to the manufacturer's plan, WST- 8 method was used to detect the content of NAD^+. The results showed that the increase trend was similar to that the expression of NMNAT3. The levels of NAD^+ in mitochondria of BMSCs modified with NMNAT3 gene increased significantly, about (346.314± 7.473) pmol/10^6 cells, compared with the control group, it increased by (2.066±0.385) times, the difference was statistically ($^*P < 0.05$). There was no significant difference between the control group and the empty virus group (Fig. 1D).

Fig. 1 Expression of NMNAT3 and the function of BMSCs after transfection

qPCR analysis of relative expression of NMNAT3 mRNA(n = 3). B,C. Western blot analysis of NMNAT3 protein expression(n = 3). D. NAD^+ levels detection of mitochondria(n = 3). E. Identification of BMSCs surface antigen by flow cytometry Note(n = 3). F. Observation of staining induced by multi-directional differentiation(n = 3). G. cell proliferation curve(n = 3). All data are presented as means ± SD, $^*P<0.05$, $^\#P$ 05. BMSC, bone marrow mesenchymal stem cells; NMNAT3, nicotinamide mononucleotide adenylyl

transferase 3; qPCR, Real-time Quantitative PCR; mRNA, messenger RNA; NAD^+, nicotinamide adenine dinucleotide; CCK-8, cell counting kit-8.

BMSCs overexpressing NMNAT3 retains its mesenchymal properties and proliferative ability

We successfully obtained a stable strains of BMSCs with high expression of NMNAT3 gene by lentivirus gene transfection, but it is unknown whether the mesenchymal characteristics and proliferation of NMNAT3 modified BMSCs are affected[38]. The surface antigens were identified by flow cytometry. The results showed that NMNAT3-modified BMSCs still expressed typical mesenchymal stem cell markers CD29, CD90 and CD106 (> 99%), while hematopoietic CD45 markers and monocyte CD11b markers showed low expression (< 1%). And its level is similar to that of the control group BMSCs (Fig. 1E, Tab. I). For its multidirectional differentiation potential[39], we used commercial kits to induce osteogenic, chondrogenic and adipogenic differentiation, and the results were shown as by alizarin red staining, alixin blue staining and oil red O (Fig. 1F). Both the genetically modified BMSCs and the control BMSCs could differentiate into osteoblasts, chondroblasts and adipocytes.

In addition, in order to understand the effect of gene modification on the proliferation of BMSCs, we continuously cultured the cells of each group and drew the growth curve by CCK-8 method. The cells in each group showed a similar proliferation trend. After a latent adaptation period of 1-2 days, a large number of cells began to proliferate into the logarithmic growth phase on the 3rd day, maintained about 3 days logarithmic growth phase, and then entered the plateau phase (Fig. 1G). Here, our study shows that after lentivirus transfection, the proliferation of genetically modified BMSCs is not affected.

The above data show that gene modification does not change the mesenchymal characteristics of BMSCs, and still retains a high ability of proliferation and differentiation.

Tab. I Flow cytometry analysis of CD labeling of stem cells in the third generation control group (Control), empty virus group (Lv-EGFP) and NMNAT3 overexpression group (Lv-NMNAT3)

Stem cell marker	Control	Lv-EGFP	Lv-NMNAT3
CD45	0.04%	0.07%△	0.06%▯
CD11b	0.38%	0.59%△	0.67%▯
CD29	99.97%	99.79%△	100.00%▯
CD90	100.00%	99.99%△	100.00%▯
CD106	99.72%	99.62%△	99.68%▯

Note: the expression of CD markers in BMSCs of each group, n=3, $^{\triangle}P$<0.05 Vs Control, $^{\square}P$<0.05 Vs Lv-EGFP.

NMNAT3 overexpression ameliorated mitochondrial damage under oxidative stress

The oxidative stress microenvironment in the transplantation area leads to the impairment of mitochondrial function of transplanted stem cells, which is the main cause of stress apoptosis of stem cells[5,8-11]. The H_2O_2 (600μM) was used to simulate the oxidative stress microenvironment[35]. The mitochondrial ultrastructure of BMSCs was observed by transmission electron microscopy after 24 hours treated with H_2O_2. The mitochondrial ultrastructure of BMSCs in H_2O_2 treated group was unclear, and the mitochondrial crest fused and broken, while the ultrastructure of BMSCs in untreated group was clear and no obvious damaging change was found (Fig. 2A). The mitochondrial membrane potential of cells was measured by JC-1 method[40,41]. Observation by laser confocal microscopy showed that the fluorescence markers of H_2O_2 treated group showed a low red and high green change, while the untreated group still showed a high red and high green. Compared with that, the mitochondrial membrane potential of cells decreased significantly in the H_2O_2 treated group (Fig. 2B,C). We used luciferase analysis to detect the ATP production of BMSCs mitochondria in each group[8]. The results showed that the mitochondrial ATP synthesis in the H_2O_2 treated group was significantly lower than that in the untreated group, Compared with the untreated group, ATP synthesis decreased by approximately (2.232±0.372) times, and the difference was statistically significant($^{*}P < 0.05$,Fig. 2D). In addition, the levels of NAD^+ and the synthesis of mitochondrial proteins PGC-1α and NRF1 in BMSCs mitochondria treated with H_2O_2 also showed the same decreasing trend. Compared with the untreated group, they respectively decreased by (1.734±0.277) times, (3.485±0.423) times and (3.727±0.308) times,and the difference was statistically significant ($^{*}P$<0.05, Fig. 2E-H). The above results indicate that the structure and function of BMSCs mitochondria are significantly impaired under oxidative stress.

In order to further study the effect of NMNAT3 on the mitochondrial function of BMSCs under oxidative stress, we continued to use H_2O_2 to treat BMSCs in NMNAT3 modified. It was observed by transmission electron microscopy that the ultrastructure of mitochondria in the overexpression group was still clear, the mitochondrial crest was continuous, and no obvious damage changes were observed (Fig. 2A). For the mitochondrial membrane potential, the immunofluorescence of BMSCs in the overexpression group showed high red and green, and there was no significant difference compared with the untreated group, but the mitochondrial membrane potential was significantly higher than that in the control group and empty virus group treated with H_2O_2, and the difference was statistically significant ($^{*}P$<0.05,Fig. 2B,C). In addition, NMNAT3 modified BMSCs showed higher levels of NAD^+, ATP, PGC-1α and NRF1 synthesis, which were (272.214±7.068) pmol/10^6 cells, (9.774±0.523) nmol/mg, (2.255±0.216) times and (2.427±0.174) times respectively. Compared with the control group and empty virus group, the mitochondrial function of BMSCs in overexpression group was significantly improved ($^{*}P$<0.05, Fig. 2D-

H). In summary, these results suggest that NMNAT3 gene overexpression can effectively improve the damage of mitochondrial structure and functions of BMSCs under oxidative stress.

Fig. 2 NMNAT3 improves mitochondrial function under oxidative stress

Observation of ultrastructure of mitochondria by transmission electron microscope (n=3). B,C. Detection of mitochondrial membrane potential by JC-1 kit (n=3). D. ATP levels detection of mitochondria (n = 3). E. NAD^+ levels detection of mitochondria(n = 3). F-H. Western blot analysis of PGC-1α and NRF1 expression in mitochondria (n = 3). All data are presented as means ± SD, $^*P$<0.05, $^\#P$□05. JC-1, 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine; ATP, Adenosine triphosphate; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator-1α; NRF1, nuclear respiratory factors 1; COX IV, Cytochrome c oxidase IV.

The anti-oxidative stress ability of BMSCs modified by NMNAT3 is enhanced

NMNAT3 modified BMSCs can effectively improve the function of mitochondria under oxidative stress, but it is not clear whether the antioxidant stress ability of modified BMSCs is enhanced. We analyzed the basic antioxidant capacity of BMSCs, and detected the level of ROS, the content of MDA and the activities of antioxidant enzymes CAT and GPX in BMSCs respectively[42,43]. As shown in figure 3, compared with the control group, the intracellular oxidative damage indicators ROS levels and MDA contents of NMNAT3 gene modified BMSCs decreased significantly under H_2O_2 simulated oxidative stress conditions, about (5.656±0.239) times and (2.664±0.185) times, while the activities of antioxidant enzymes CAT and GPX increased significantly, respectively (11.365±0.577) U/mg and (14.629±0.729) U/mg, the difference was statistically significant ($^*P$<0.05, Fig. 3A-E). In addition,the senescence-related SA-β-Gal activity and apoptosis in BMSCs of each group were also detected[44]. In the NMNAT3 overexpression group, the SA-β-Gal activity decreased, the percentage of SA-β-Gal positive cells decreased by (3.152±0.756) times, and the number of apoptotic cells also decreased significantly, while the control group and empty virus group under the same conditions did not show the same change trend, the difference was statistically significant ($^*P$<0.05, Fig. 3F-H). The above results showed that the antioxidant stress ability of BMSCs modified by NMNAT3 was enhanced.

Fig. 3 NMNAT3 overexpression enhances the antioxidant stress ability of BMSCs

A,B. DCFH-DA fluorescence probe detection of ROS (n = 3). C. MDA in BMSCs Content detection. D. Analysis of CAT activity in BMSCs. E. Analysis of GPx activity in BMSCs. F,G. Detection of β-gal activity by β-gal staining (n=4). H. TUNEL/DAPI staining method for detecting apoptosis (n = 4). All data are presented as means ± SD, $^*P$ <0.05, $^\#P$□0.05. DCFH-DA, 2',7'-Dichlorofluorescin diacetate; ROS, reactive oxygen species; MDA, Malondialdehyde; CAT, Catalase; GPx, Glutathione peroxidase; SA-β-Gal, senescence associated-β-galactosidase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole.

Overexpression of NMNAT3 enhances the activity of Sirt3 under oxidative stress

At the molecular level, the mechanism by which NMNAT3 overexpression modified BMSCs and increases NAD^+ levels in mitochondria to improve mitochondrial function and enhance its antioxidant stress ability is not clear. Sirt3 is an important protein that regulates mitochondrial metabolism and oxidative homeostasis, and its activity is closely related to the levels of NAD^+[26,45,46]. Does the decrease of NAD^+ levels caused by mitochondrial dysfunction under oxidative stress affect Sirt3 catalytic activity, limit its regulation and improve mitochondrial function? By increasing the expression of NMNAT3 and increasing the levels of NAD^+, does it affect the catalytic activity of Sirt3, and then improve the mitochondrial function? Therefore, we quantitatively analyzed the expression and activity of Sirt3 by Western Blot and colorimetry. The expression levels of Sirt3 in BMSCs of NMNAT3 overexpression group was not significantly increased, but its activity was significantly higher than that of the control group and empty virus group, approximately (1.545±0.073) times. ($^*P$<0.05, Fig. 4A,B). Further detection by Co-IP method showed that, compared with the empty virus group, the acetylation levels of Idh2 and FOXO3a in BMSCs modified by NMNAT3 gene decreased by (2.746±0.117) times and (2.625±0.146) times respectively, the activities of ATP synthase and Mn-SOD increased by (1.551±0.098) times and (1.829±0.083) times, and the ratio of GSH/GSSH increased by (1.715±0.139) times, and all differences were statistically significant ($^*P$<0.05, Fig. 4A,C-G). These results indicate that NMNAT3 increases the levels of NAD^+ in mitochondria and plays a role in the activation of Sirt3. However, we are not sure that the abovfresulte changes are NAD^+-Sirt3 dependent, and we can not rule out that it may be caused by the direct regulation of NMNAT3-NAD^+. Therefore, we further used the selective Sirt3 inhibitor (3-TYP) to inhibit the activity of Sirt3[47]. The results showed that the addition of Sirt3 inhibitors increased the acetylation levels of Idh2 and FOXO3a in mitochondria of NMNAT3 modified BMSCs, while the levels of GSH, ATP synthase and Mn-SOD activity decreased, and the effect of NMNAT3 was completely blocked (Fig. 4A-G). Here, our results further support that NMNAT3 through the NAD^+-Sirt3 axis is a potential mechanism for improving mitochondrial function and enhancing the antioxidant stress ability of BMSCs.

Fig.4 NMNAT3 enhances the activity of Sirt3 under oxidative stress

A,C,D. Western blot analysis of Sirt3, IDH2 and FOXO3a levels in BMSCs. Acetylated IDH2 and FOXO3a were isolated by immunoprecipitation with anti-IDH2 and anti-FOXO3a antibody followed by western blotting with anti-acetyl-lysine antibody (n=3). B. Detection of Sirt3 relative activity in BMSCs. E. Analysis of ATP synthase activity in BMSCs. F. Analysis of Mn-SOD activity in BMSCs. G. Detection the ratio of GSH/GSSH in BMSCs. All data are presented as means ± SD, $^*P$ <0.05, $^\#P$ 0.05. Sirt3, silent mating type information regulation 2 homolog 3; Idh2, isocitrate dehydrogenase 2; FOXO3a, Forkhead-box protein O3a; Mn-SOD, manganese superoxide dismutase; GSH/GSSH, reduced glutathione/oxidized glutathione.

Discussion

The low survival rate of stem cell transplantation in vivo is related to the dysfunction of stem cell mitochondria caused by oxidative stress microenvironment in the transplantation area, which is mainly manifested by the decrease of NAD^+ levels in mitochondria[48,49]. Many studies have shown that decreased NAD^+ levels can inhibit the activity of NAD^+ -dependent enzymes in mitochondrial oxidative phosphorylation, TCA cycle and glycolysis, thereby reducing the production of ATP and damage the structure and function of mitochondria[17,50,51]; the decrease of NAD^+ levels can also lead to the disturbance of cell signal molecular pathways, resulting in cell aging and apoptosis[14,52]. Therefore, maintaining NAD^+ homeostasis is an effective strategy to combat mitochondrial disorders and improve cell survival[15,53]. SonMJ et al. found that NMNAT3 overexpression is an ideal method to increase the levels of NAD^+ in cell mitochondria[33]. In this study, we also found that the NAD^+ levels in mitochondria of BMSCs modified by NMNAT3 was significantly higher than the control group (2.895 ± 0.413 times, $P < 0.05$), and the decrease of NAD^+ levels caused by oxidative stress was recovered. It also effectively ameliorated the mitochondrial structural damage of BMSCs under oxidative stress, increased mitochondrial membrane potential, increased ATP and mitochondrial protein synthesis (Fig. 2). Compared with the control group, the mitochondrial structure and function of BMSCs modified by NMNAT3 were significantly improved, which indicated that under oxidative stress, NMNAT3 played a beneficial role in improving the damage of mitochondrial structure and function by restoring the levels of NAD^+ in mitochondria. In addition, under oxidative stress, the activities of antioxidant enzymes (CAT and GPx) in BMSCs modified by NMNAT3 were significantly higher than that in the control group ($P < 0.05$), the content of MDA and ROS reduced significantly (Fig. 3), which delayed cell aging and reduced apoptosis. This result can be attributed to the improvement of their ability of antioxidant stress.

Here, we also provide molecular insights into how NAD^+ regulates mitochondrial function. In recent years, the deacetylase Sirtuins protein family has been widely concerned by researchers[47,54–56], in which Sirt3 plays an indispensable role in regulating mitochondrial energy metabolism and oxidative stress [45,46,57–59]. The biological activity of Sirt3 depends on the levels of NAD^+, and NAD^+ levels decrease during mitochondrial dysfunction, which seriously affects the activity of Sirt3 and limits its role in regulating mitochondrial function[26,28,60–63]. In this study, it was found that under oxidative stress, the expression of Sirt3 protein did not change significantly, but significant differences in its activity, which may be related to low NAD^+ levels, While BMSCs modified by NMNAT3 overexpression can significantly increase Sirt3 activity after increasing NAD^+ levels (1.545 ± 0.073 times, $P < 0.05$), and significantly decrease the acetylation level of Sirt3-dependent deacetylation-related proteins (Idh2, FOXO3a) in mitochondria. The content and activity of effector proteases (GSH, ATP synthase, Mn-SOD) were increased (Fig. 4), which shows that under oxidative stress conditions, NMNAT3 significantly increased Sirt3 activity and improved the function of mitochondria by increasing the levels of NAD^+. In order to further demonstrate the key role of Sirt3 in the effect of mitochondrial function, we treated with Sirt3 inhibitor and completely blocked this effect. The NMNAT3-NAD^+ did not directly decrease the acetylation

levels of mitochondrial proteins (Idh2, FOXO3a) or increase the activity of effector proteases (GSH, ATP synthase, Mn-SOD) (Fig. 4). The results show that this change is not directly affected by NMNAT3-NAD^+, but NAD^+-Sirt3 is dependent. Our results support that the NMNAT3-NAD^+-Sirt3 axis is a potential mechanism to improve mitochondrial function and enhance anti-oxidant stress in BMSCs.

Conclusions

In summary, We have demonstrated that the NMNAT3 gene has a positive effect on improving mitochondrial dysfunction of BMSCs under oxidative stress and enhancing the ability of antioxidant stress in vitro cell experiments, and proved that NMNAT3-NAD^+-Sirt3 axis is the potential mechanism of improving mitochondrial function. This study provides a new strategy to solve the problem of survival rate of stem cell transplantation and has great significance to promote the clinical transformation of stem cell therapy. However, it is unclear whether NMNAT3 has the same effect on the complex environment in vivo[64,65]. Future research may focus on this issue to further evaluate the role of NMNAT3 in vivo and provide experimental evidence for comprehensively elucidating the clinical application of NMNAT3.

Abbreviations

3-TYP
3-(1H-1,2,3-triazol-4-yl) pyridine
ATP
Adenosine triphosphate
BMSCs
bone marrow mesenchymal stem cells
CAT
Catalase
CCK-8
cell counting kit-8
COX IV
Cytochrome c oxidase IV
DCFH-DA
2’,7’-Dichlorofluorescin diacetate
DAPI
4',6-diamidino-2-phenylindole
FOXO3a
Forkhead-box protein O3a
GPx
Glutathione peroxidase
GSH/GSSH

reduced glutathione/oxidized glutathione
Idh2
isocitrate dehydrogenase 2
JC-1
5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine
MDA
Malondialdehyde
Mn-SOD
manganese superoxide dismutase
mRNA
messenger RNA
NMNAT3
nicotinamide mononucleotide adenylyl transferase 3
NAD^+
nicotinamide adenine dinucleotide
NRF1
nuclear respiratory factors 1
PGC-1α
Peroxisome proliferator-activated receptor gamma coactivator-1α
ROS
reactive oxygen species
RT-qPCR
Real-time Quantitative PCR
Sirt3
silent mating type information regulation 2 homolog 3
SA-β-Gal
senescence associated-β-galactosidase
TUNEL
terminal deoxynucleotidyl transferase dUTP nick end labeling

Declarations

Acknowledgments

Not applicable.

Funding

the Regional Science Foundation of National Natural Science Foundation of China (Grant Nos. 81860387); the Youth Science Foundation of National Natural Science Foundation of China (Grant Nos. 81902226); the Guizhou Provincial Science and Technology Department–Guizhou Medical University Joint Fund (Grant Nos. [2017]7197); the Science and Technology Department of Guiyang (Grant Nos. GY2016-3); the Science and Technology Fund of Guizhou Provincial Department of Health (Grant Nos. gzwjkj2019-1-135)

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Contributions

, W.X.P. and F.Z. generated the hypothesis and concept of the manuscript,T.W. and F.Z. designed experiments, performed most of the experiments.T.W. and J.Z. performed the mitochondrial studies,T.W.,F.Z. and W.T.D. performed cell experiments, western-blots, and PCRs.L.W. and C.Y. analyzed and interpreted the data, T.W. edited and wrote the manuscript. X.B.T., Y.L.L and Y.K.G. revised the manuscript.All authors contributed to writing the manuscript.

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Ethics declarations

Ethics approval and consent to participate

All animals are approved by the Experimental Animal Bioethics Committee of Guizhou Medical University (Grant No.1900590), and all procedures are carried out in strict accordance with the guidelines for the Care and use of Experimental Animals issued by the National Institutes of Health (NIH publication No. 85-23, 1996 revised).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

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

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Figures

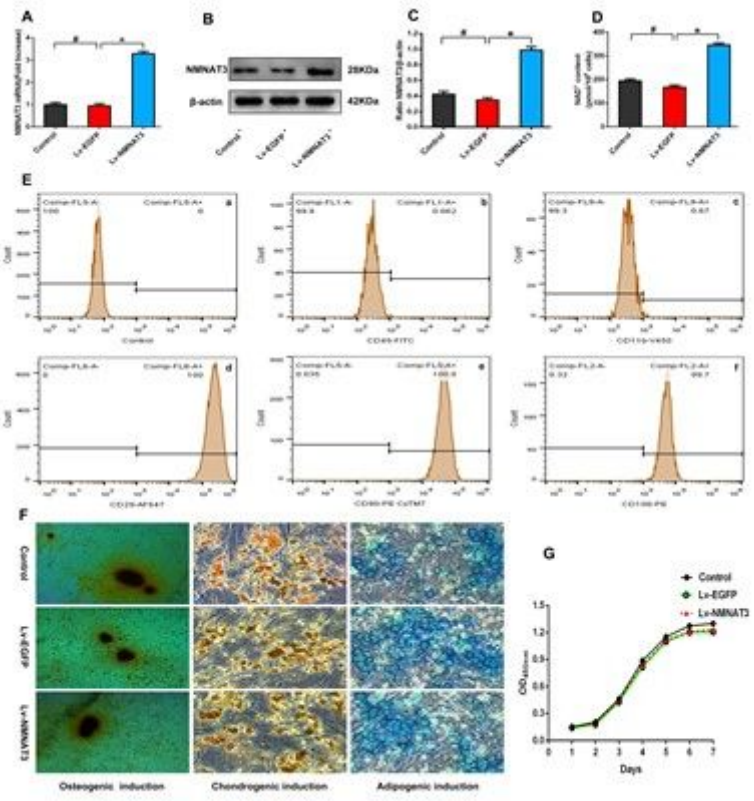

Figure 1

Expression of NMNAT3 and the function of BMSCs after transfection A. qPCR analysis of relative expression of NMNAT3 mRNA(n = 3). B,C. Western blot analysis of NMNAT3 protein expression(n = 3). D. NAD+ levels detection of mitochondria(n = 3). E. Identification of BMSCs surface antigen by flow cytometry Note(n = 3). F. Observation of staining induced by multi-directional differentiation(n = 3). G. cell proliferation curve(n = 3). All data are presented as means ± SD, *P <0.05, #P□0.05. BMSC, bone marrow mesenchymal stem cells; NMNAT3, nicotinamide mononucleotide adenylyl transferase 3; qPCR, Real-time Quantitative PCR; mRNA, messenger RNA; NAD+, nicotinamide adenine dinucleotide; CCK-8, cell counting kit-8.

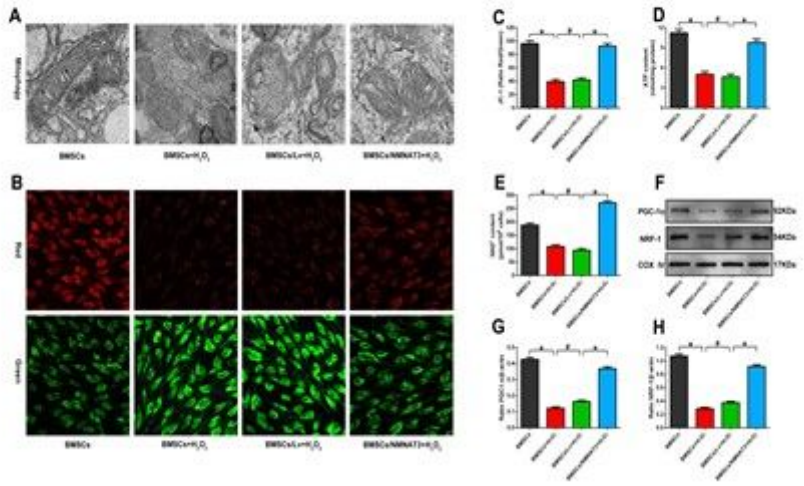

Figure 2

NMNAT3 improves mitochondrial function under oxidative stress A. Observation of ultrastructure of mitochondria by transmission electron microscope (n=3). B,C. Detection of mitochondrial membrane potential by JC-1 kit (n=3). D. ATP levels detection of mitochondria (n = 3). E. NAD+ levels detection of mitochondria(n = 3). F-H. Western blot analysis of PGC-1α and NRF1 expression in mitochondria (n = 3). All data are presented as means ± SD, *P <0.05, #P□0.05. JC-1, 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine; ATP, Adenosine triphosphate; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator-1α; NRF1, nuclear respiratory factors 1; COX IV, Cytochrome c oxidase IV.

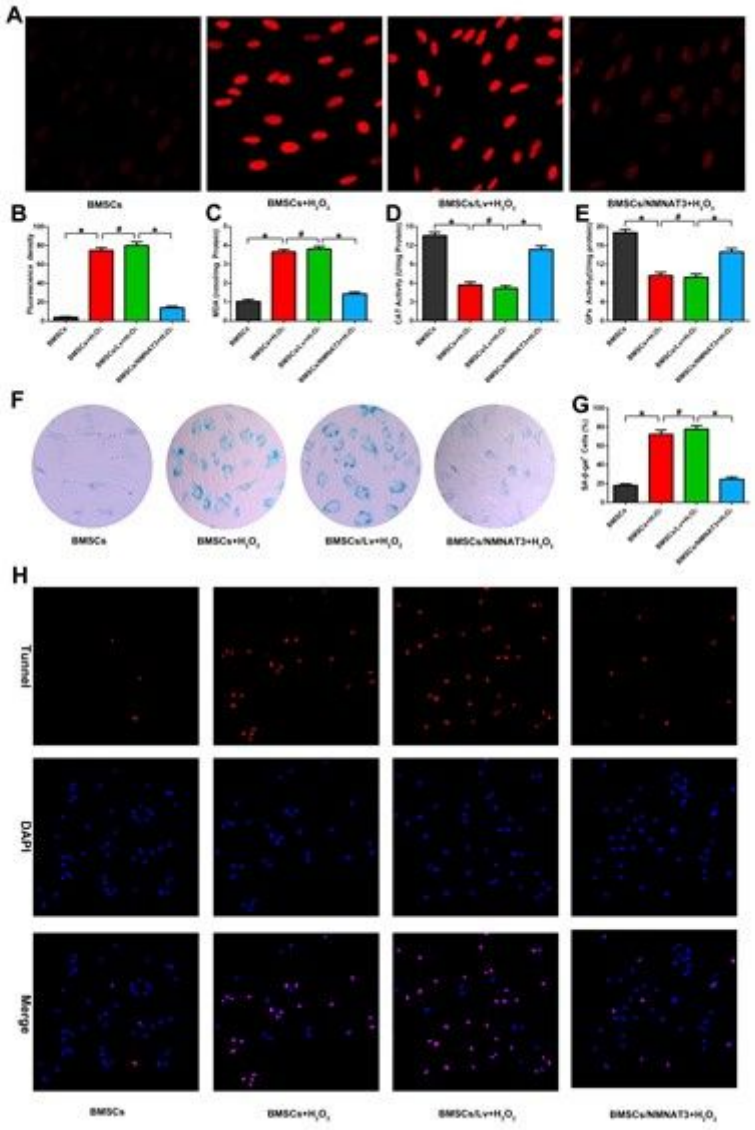

Figure 3

NMNAT3 overexpression enhances the antioxidant stress ability of BMSCs A,B. DCFH-DA fluorescence probe detection of ROS (n = 3). C. MDA in BMSCs Content detection. D. Analysis of CAT activity in BMSCs. E. Analysis of GPx activity in BMSCs. F,G. Detection of β-gal activity by β-gal staining (n=4). H. TUNEL/DAPI staining method for detecting apoptosis (n = 4). All data are presented as means ± SD, *P <0.05, #P□0.05. DCFH-DA, 2',7'-Dichlorofluorescin diacetate; ROS, reactive oxygen species; MDA, Malondialdehyde; CAT, Catalase; GPx, Glutathione peroxidase; SA-β-Gal, senescence associated-β-galactosidase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole.

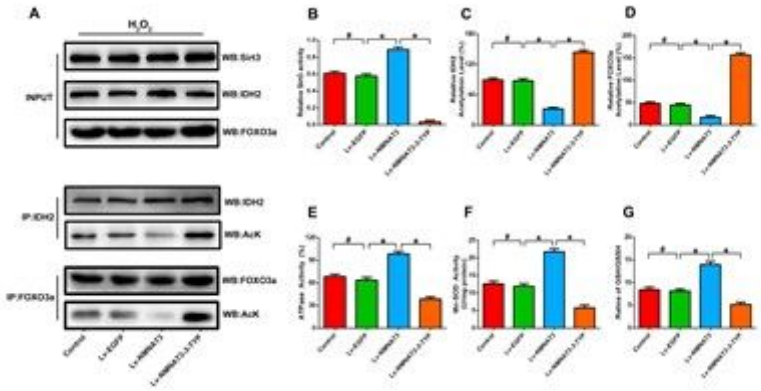

Figure 4

NMNAT3 enhances the activity of Sirt3 under oxidative stress A,C,D. Western blot analysis of Sirt3, IDH2 and FOXO3a levels in BMSCs. Acetylated IDH2 and FOXO3a were isolated by immunoprecipitation with anti-IDH2 and anti-FOXO3a antibody followed by western blotting with anti-acetyl-lysine antibody (n=3). B. Detection of Sirt3 relative activity in BMSCs. E. Analysis of ATP synthase activity in BMSCs. F. Analysis of Mn-SOD activity in BMSCs. G. Detection the ratio of GSH/GSSH in BMSCs. All data are presented as means ± SD, *P <0.05, #P□0.05. Sirt3, silent mating type information regulation 2 homolog 3; Idh2, isocitrate dehydrogenase 2; FOXO3a, Forkhead-box protein O3a; Mn-SOD, manganese superoxide dismutase; GSH/GSSH, reduced glutathione/oxidized glutathione.