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Experimental Induction of Myogenic Differentiation in Rabbit Bone Marrow Mesenchymal Stem Cells Using Different Concentrations of 5-Azacytidine

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Abstract

Purpose: To Bone Marrow Mesenchymal Stem Cells different concentrations Azacytidine

Method: BMSCS from adult New Zealand male white rabbits were expanded and cultured *in vitro*. Cells were induced with 0, 5, 10, 20, and 40 μmol/L 5-Aza for 24 hours, and these groups were denoted groups A,B,C,D, and E. Group A served as the control group, and groups B, C, D, and E were the experimental groups. The absorbance of the cells at 570 nm was measured using the MTT method, which is commonly used for assessing cell viability. On days 1, 3, 5, 7 and 9 after induction, the proliferation and morphological changes of BMSCS were observed using an inverted light microscope. On day 9 of cultivation, we digested and collected the cells and prepared slides of the cells. The expression of actin was assessed by immunocytochemistry, and the percentage of actin expression was determined through a semi quantitative analysis of fluorescence. Quantitative RT-PCR was used to detect the mRNA expression of troponin and myosin.

Results: The flow cytometry results showed that the surface markers CD34, CD44 and CD90 were expressed at percentages of 2.52%, 94.6% and 92.8%, respectively. The 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-tetrazolium bromide (MTT) assay revealed that cell viability was significantly lower in group E than in group A ($p < 0.001$). On day 9, the cell morphology changes observed in group C were the most obvious. Actin expression was detected in groups A, B and C by immunohistochemistry, and semi quantitative fluorescence showed that group C exhibited significantly higher myogenic differentiation in BMSCS than group A ($p=0.034$). On day 9 after induction, troponin and myosin mRNA expression levels were higher in group C than in group A ($p=0.04$ and $p < 0.001$, respectively), as demonstrated by quantitative real-time PCR.

Conclusion: Different concentrations of 5-Aza exert different effects on the proliferation and myogenic differentiation of fifth-generation BMSCS. In addition, 10 μmol/L, 5-Aza significantly promoted myogenic differentiation, whereas 40 μmol/L 5-Aza exerted a certain toxic effect on BMSC proliferation.

Keywords: 5-Azacytidine; Bone marrow mesenchymal stem cells; Proliferation; Myogenic differentiation; Actin; Troponin; Myosin

Abbreviations: MTT: 3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2-Tetrazolium Bromide; BMSCS: Bone Marrow-Derived Mesenchymal Stem Cells; 5-Aza: 5-Azacytidine; PBS: Phosphate Buffered Saline; DMEM: Dulbecco's Modified Eagle's Medium; FACS: Fluorescence-Activated Cell Sorting; PCR: Polymerase Chain Reaction; SPSS: Statistical Package for the Social Sciences; HBSS: Hanks Balanced Salt Solution

Introduction

Bone marrow mesenchymal stem cells (BMSCS) are pluripotent stem cells that can undergo multipotential differentiation and are utilized as seed cells in many stem cell experiments [1-2]. BMSCS are clonogenic, no hematopoietic stem cells that exist in the bone marrow and can differentiate into multiple mesoderm-type cell lineages, such as osteoblasts, chondrocytes, and endothelial cells, as well as non-mesoderm-type lineages [3-6]. Moreover, BMSCS exhibit stable characteristics and can be obtained from many sources because they continue to exhibit multidirectional differentiation potential after continuous subculture. Thus these cells have promising application prospects in tissue engineering [7]. In 1995, Wakitani et al. [8] were the first to report that 5-Aza can induce BMSCS to undergo myogenic differentiation, and currently, 5-Aza is used mainly used to induce the differentiation of stem cells into muscle cells [9]. However, although the relevant mechanism has not been confirmed, it might be related to the demethylation of regulatory cell housekeeping genes by 5-Aza [10-12]. Few studies have shown the ability of rabbit BMSCS to differentiate into myogenic cells in the presence of 5-Aza, although a rabbit model is urgently needed. Therefore, this study aimed to assess the effects of different concentrations of 5-Aza on the proliferation and

myogenic differentiation of rabbit BMSCS, which could aid further research.

We hypothesized that different concentrations of 5-Aza induce rabbit BMSCS but exert different effects on their myogenic differentiation *in vitro*. To test this hypothesis, we induced rabbit BMSCS with different concentrations of 5-Aza.

Materials and Methods

All animals received humane care in compliance with the Guidelines for the Care and Use of Laboratory Animals by the Department of Science and Technology of the People's Republic of China (2006). Primary BMSCS were extracted from a healthy adult New Zealand white rabbit that was provided by the Jiangsu University Experimental Animal Center of China.

Preparation and culture of rabbit BMSCS

An adult New Zealand male white rabbit was weighed and a 0.6% sodium pentobarbital solution (4 mg/kg) was injected into the rabbits' ear veins for general anesthesia. The hair on both sides of the anterior superior iliac spine was removed. These surfaces were then disinfected with iodophor, and a hole was introduced with a dowel. A 16-gauge bone marrow puncture needle was used to penetrate the anterior superior iliac spine. Five milliliters of rabbit bone marrow was extracted and immediately added to a sterile centrifuge tube containing heparin, and the tube was shaken as the bone marrow was added. An equal volume of lymphocyte separation solution (Tianjin Haoyang Biotechnology Comparison, China) was added to the tube containing the bone marrow, and the tube was centrifuged at 2000 rpm for 20 minutes. The intermediate white flocculent nucleated cell layer was pipetted five times into D-Hanks Balanced Salt Solution (HBSS, Gibco Company, St Louis, MO, USA), and the mixture was centrifuged at 1200 rpm for 10 minutes. After centrifugation, the supernatant of the upper layer was discarded, and the cells were seeded into a 25 cm^2 plastic flask (Costar, USA) with DMEM, 10% Fetal Bovine Serum (FBS, Gibco Company, St Louis, MO, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco Company, St Louis, MO, USA) and incubated at 37°C with 5% CO_2. After 24 hours, non-adherent cells were removed by replacing the DMEM with FBS. The medium was replaced with fresh medium with FBS every 3 days, and passage-5 BMSCS were used for the experiments.

Induction of differentiation and division into BMSC groups

BMSCS were induced with 0, 5, 10, 20, and 40 μmol/L 5-Aza, and these groups were denoted groups A, B, C, D, and E. Group A served as the control group, and groups B, C, D, and E were the experimental groups. After induction for 24 hours in a 5% CO_2 saturated humidity incubator at 37°C, the DMEM medium was discarded. The 25-cm^2 plastic flasks were washed three times with HBSS, and the medium was replaced with fresh DMEM with FBS every 3 days. Two 25-cm^2 plastic flasks were included in each group.

Flow cytometry analysis of surface antigens of BMSCS

Passage-3 adherent cells were treated with 0.25% trypsin (Gibco, USA) and washed twice with PBS. The cells were incubated with rabbit anti-CD34, rabbit anti-CD44, and rabbit anti-CD90 antibodies (Invitrogen, USA, and Gibco, USA) overnight at 4°C. Unbound antibodies were removed by washing three times with PBS. After washing, the cells were incubated for 45 minutes at room temperature in the dark with Cy3-labeled secondary anti-goat/anti-rabbit antibody and re-suspended in PBS for FACS analysis. At least 1×10^6 cells per sample were analyzed with a flow cytometer (BD FACSVerse, USA). CELL Quest software was used for the analysis.

Assessment of cell viability by MTT assay

Cell viability was quantitatively determined using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-tetrazolium bromide (MTT) method. MTT is a yellow tetrazolium dye that responds to metabolic activity, and the reductases in living cells reduce MTT from a pale yellow color to dark blue formazan crystals. Cells were seeded at 1×10^4/well in 100 μL of Dulbecco`s Modified Eagle Media (DMEM) with Fetal Bovine Serum (FBS) in 96-well plates (Thermo Scientific, USA). After attachment, the cells were exposed to 0, 5, 10, 20 and 40 μmol/L, 5-Aza, and each concentration was added to five replicate wells. After 24 hours, the culture medium was changed to FBS, and the cells were tested on days 1, 3, 5, 7, and 9, at which point 100 μL of MTT (Beyotime Biotechnology, China) was added to each well. The plates were incubated for 4 hours at 37°C. The supernatants were then removed, and 200 μL of Dimethyl Sulfoxide (DMSO, Merck, Germany) was added to each well to dissolve the blue crystals. The absorbance (Optical Density (OD)) at 570 nm was read using a microplate reader (Biotek, USA).

Observation of cell morphology using an inverted microscope

The morphological changes of each group of cells on days 1, 3, 5, 7, and 9 were observed under an inverted photomicroscope (Leica, Germany).

Immunocytochemistry and semi-quantitative analysis of the fluorescence of actin expression

Nine days after induction, the cells from groups A, B and C were digested and collected. The cells were then seeded at 3×10^4 cells/well in a 12-well culture plate (with prepositioned cell slides) and incubated at 37°C in an incubated with 5% CO_2 saturated humidity for 48 hours. Six cell slides were obtained for each of the groups (A, B and C). The slides were removed, fixed in 4% paraformaldehyde for 30 minutes, permeabilized with 0.1% Triton-100 (Merck, Germany) for 30 minutes at room temperature, blocked with 5% BSA (Sigma-Aldrich, USA) in Tween 20 (Merck, Germany) and PBS (Gibco, USA) for 1 hour at room temperature, then incubated overnight at 4°C in the presence of α-SMA (1: 200) (Proteintech, USA), and blocked with 1% BSA. A Cy3-labeled secondary antibody (1: 100) (Proteintech, USA) diluted with 1% BSA was added to each slide, and the slides were then incubated for 1 hour at 37°C. PBS was added to each slide for 5 minutes, and the liquid was discarded; this step was repeated five times. Hoechst 33258 stain (Beyotime Biotechnology, China) was added to each slide, and the slides were incubated for 30 minutes at room temperature in the dark. After washing, the slides were sealed, and photomicrographs were taken using a fluorescence microscope (Leica, Germany).

A common laser-scanning common microscope can quantify the confocal fluorescence of fluorescently labeled tissue specimens for confocal fluorescence and can display the fluorescence changes in the Z-axis direction. Photographs were obtained using ImageJ software (Rawak Software, Germany), and the data were statistically analyzed using PrismDemo software (GraphPad Software, USA).

Quantitative RT-PCR detection of the mRNA expression of troponin and myosin

Intracellular total RNA extraction: Cells were washed twice with HBSS, and the supernatant liquid was discarded. The cells were then incubated with 1 ml of TRIzol (Invitrogen, USA) for 5 minutes on an ultraclean workbench (Suzhou Antai Science and Technology Co., Ltd., Suzhou China). After 0.2 ml of chloroform (Shanghai Medicine, China) was added, the cells were shaken for 15 seconds, incubated for 3 minutes at room temperature and centrifuged at 12,000 rpm and 4°C for 10 minutes (Sigma Centrifuge, Germany). The supernatant liquid was discarded, and 0.5 ml of isopropanol was added. The mixture was then mixed well for 30 minutes on ice and centrifuged at 12,000 rpm and 4°C for 4 minutes, and the supernatant liquid was discarded. The precipitate was washed by adding of 1 ml of 75% ethanol, and the mixture was centrifuged at 7500 rpm and 4°C for 5 minutes. The supernatant liquid was discarded, and the precipitate was dried for 5 minutes on an ultraclean workbench. An appropriate amount of RNase-free H_2O (Biosharp, China) was added until the precipitate was dissolved.

cDNA synthesis: The reverse-transcription reaction system was prepared using a reverse transcription kit (Thermo Scientific, USA) according to the instructions.

Polymerase chain reaction: Primer synthesis: The primers used for reverse-transcription PCR were designed and synthesized using Primer Premier 5.0 software (Shanghai Biotech, China), and the housekeeping gene GAPDH was used as an internal reference as, shown in Table 1.

Primer name	Primer sequence
Troponin (F)	GCTAGCCTCTGGATTTGACG
Troponin (R)	ACCAGGACCAGAGGAAACCT
Myosin (F)	GCCCAGTACCTCCGAAAGTC
Myosin (R)	GCCTTAACATACTCCTCCTTGTC
GAPDH (F)	ACCTGACCTGCCGCCTGGAGAAAGC
GAPDH (R)	GGAGACGACCTGGTCCTCGGTGTAG

Table 1: Primers for troponin and myosin.

Real-time-PCR reaction system: The real-time PCR reaction system was prepared using a SYBR Green PCR kit (Thermo Scientific, USA) according to the instructions.

PCR amplification conditions: The reaction system was placed into the real-time machine for detection. Two duplicate wells were used for each group, and the following temperature program was used: 94°C for 10 minutes and 40 cycles of 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 seconds.

Data analysis: After PCR amplification, the real-time PCR system automatically analyzed the results, adjusted the threshold and baseline according to the negative control to determine the Cycle Threshold (Ct) value of each specimen, and determined whether the Ct value was valid according to the melting curve. The results were derived, and the difference in the expression of the target gene between the control group and each experimental group was analyzed using the $2^{-\Delta\Delta Ct}$ method. First, ΔCt was calculated as the Ct of the target gene-the Ct of the internal reference gene, and the ΔCt of the control was similarly calculated. The ΔCt was then subtracted from the ΔCt of the control to obtain the ΔΔCt value, and the $2^{-\Delta\Delta Ct}$ value of each group was calculated to determine the relative expression level of the target gene in each group.

Statistical analysis

SPSS 20 software for Windows (SPSS Inc., Chicago, IL, USA) was used to record the data and for the statistical analyses. The data are presented as the means ± standard deviations (SDs) of various measurements. Student's t test was used to compare a single experimental mean with the control mean, and $p < 0.05$ was considered statistically significant.

Results

Expression of BMSC surface markers

The identity of the BMSCS was confirmed using specific membrane markers, and the results showed that the cells prominently expressed CD44 and CD90 (94.6% and 92.8% positive, respectively) and did not express CD34 (2.52% positive). This information was published in a previous article.

Cell viability

The MTT results showed that the cell viability was lower in group B than in group A, as presented in Table 2. No statistically significant difference in the myogenic differentiation rate was found between groups A and B (p=0.076). Cell viability was lower in group C than in group A, as presented in Table 2, and no statistically significant difference in the myogenic differentiation rate was found between these groups (p=0.052). The cell viability of group D was substantially lower than that of group A, as presented in Table 2. A statistically significant difference in the myogenic differentiation rate was detected between groups A and D (p=0.01). Cell viability was obviously lower in group E than in group A, as shown in Table 2, and a statistically significant difference in the myogenic differentiation rate was observed between these groups (p <0.001).

Group	Day1	Day 3	Day 5	Day 7	Day 9
A	0.142 ± 0.023	0.216 ± 0.035	0.373 ± 0.022	0.591 ± 0.267	0.729 ± 0.033
B	0.137 ± 0.040	0.206 ± 0.015	0.370 ± 0.035	0.537 ± 0.071	0.707 ± 0.071
C	0.134 ± 0.044	0.201 ± 0.018	0.366 ± 0.025	0.520 ± 0.056	0.640 ± 0.035
D	0.129 ± 0.074	0.193 ± 0.063	0.234 ± 0.072	0.291 ± 0.148	0.333 ± 0.160
E	0.114 ± 0.027	0.195 ± 0.027	0.207 ± 0.029	0.243 ± 0.069	0.285 ± 0.083
The p values for the differences between groups A and B, C, D and E were as p=0.076, p=0.052, p=0.01 and p <0.001 respectively.					

Table 2: Evaluation of the effect of 5-Aza on the viability of BMSCS through the MTT assay (n=5, $\bar{x} \pm s$).

On days 1 and 3, no obviously significant change in cell viability was observed among the groups. On day 5, the cell viability of groups A, B and C was significantly increased, whereas that of groups D and E showed a slow increases. On day 7, the cell viability of groups B and C was lower than that of group A but significantly higher than that of groups D and E (Figure 1). On day 9, the cell viability of all the groups continued to increase, and groups A and E exhibited the highest and lowest viability, respectively (Figure 1).

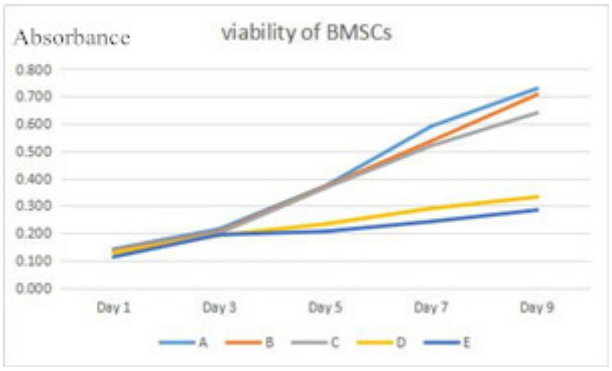

Figure 1: Viability of BMSCS.

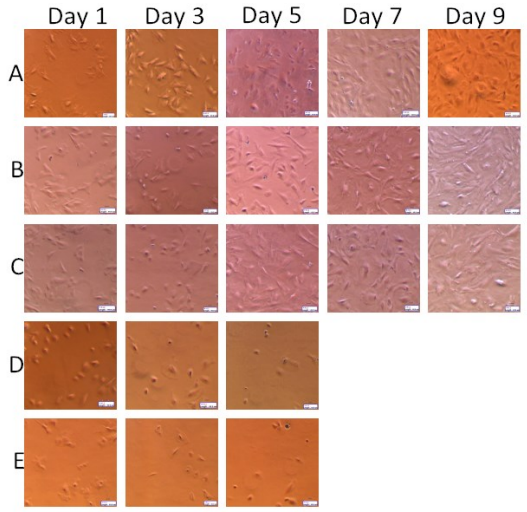

Figure 2: (A-E) Morphological changes in BMSCS.

Morphological changes in BMSCS

After 9 days of cultivation, the number of cells in group A increased, but their cellular morphology did not show any obvious changes (Figure 2A). In contrast, at this same time point, the number of cells in group B increased, and some cells became thicker and longer (Figure 2B). Moreover, after 9 days of induction, the number of cells in group C increased, and some cell morphology changes, including a gradual decrease in volume gradually and a fusiform or irregular shape, were observed (Figure 2C). In addition, the cells were arranged in a regular manner, and the growth of long spindle-shaped monocytes was observed in parallel. After 5 days of cultivation, the cells in group D exhibited slow growth and no obvious changes in morphology (Figure 2D), whereas some of the cells in group E had died, and the rest grew slowly and showed no obvious changes in morphology (Figure 2E).

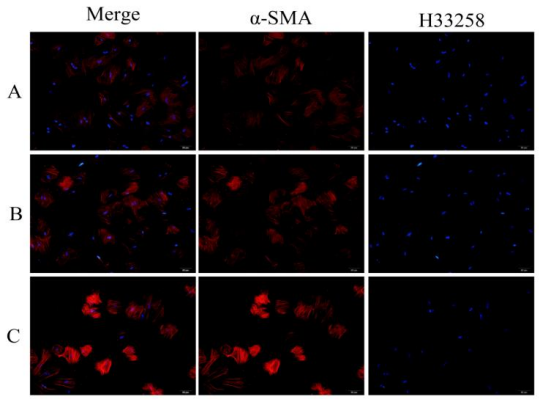

Figure 3: (A-C) Immunocytochemical characteristics of differentiated BMSCS.

Myogenic differentiation rate

Three slides from groups A, B and C were randomly selected. A semi-quantitative analysis of their fluorescence showed that group C exhibited the highest rate of myogenic differentiation. The myogenic differentiation rate of group B was higher than that of group A, as presented in Table 3, and no statistically significant difference in the myogenic differentiation rate was found between these groups A and B (p=0.102). However, the myogenic differentiation rate of group C was significantly higher than that of group A, as shown in Table 3, and the difference was statistically significant (p=0.034)

Group	Differentiation rate of BMSCS
A	0.026 ± 0.023
B	0.447 ± 0.050
C	0.591 ± 0.108
The P values of the comparisons of groups A with B and C were P=0.102 and P=0.034, respectively.	

Table 3: Myogenic differentiation rate of BMSCS in groups A, B and C (n=3, $\bar{x} \pm s$).

Group	Troponin	Myosin
A	1.000 ± 0.015	1.002 ± 0.093
B	2.130 ± 0.104	3.006 ± 0.440
C	3.426 ± 0.218	4.332 ± 0.021
The P values of the comparisons of groups A with B and C were P=0.102 and P=0.034, respectively.		

Table 4: Troponin and Myosin gene expression in groups A, B and C (n=2, $\bar{x} \pm s$).

Troponin and myosin gene expression

The quantitative RT-PCR results showed higher mRNA expression levels of troponin and myosin in group B than in group A, as presented in Table 4. A statistically significant difference in troponin mRNA expression was found between groups A and B (p=0.04), but no statistically significant difference in the mRNA expression level of myosin was detected between these groups A and B (p=0.24). The troponin and myosin mRNA expression levels were significantly higher in group C than in group A, as shown in Table 4 (p=0.04 and p <0.001 respectively).

Discussion

Due to their pluripotent differentiation ability, BMSCS are used widely in tissue engineering, and the previous research on stem cell treatments for muscle damage has provided new treatment ideas. Skeletal muscle consists of a terminally differentiated cell type with limited regenerative capacity [13]. Therefore, the focus of research in stem cell treatments and regenerative medicine research is the identification of an alternative source of stem cells. Stilhano et al. [14] performed a systematic review and found that the combination of the properties of stem cells with growth factors might be a future alternative therapy for muscle injury that could achieve faster recovery. Stocum et al. [15] found that BMSCS can differentiate into muscle cells after their transplantation into muscle cell populations. BMSC differentiation is a process in which a variety of cytokine interactions induce a series of complex physiological and biochemical reactions in cells. These mechanisms of action remain unclear, and a more effective strategy for inducing the differentiation of BMSCS into muscle cells might be a promising treatment for patients with clinical muscle damage. Once favorable cells for seeding are obtained, it is important to control the direction of their differentiation and increase the efficiency of their differentiation. In our experiment, we attempted to identify a suitable concentrations of 5-Aza for inducing the myogenic differentiation of rabbit BMSCS.

Currently, many experiments use the rabbit degenerated rotator cuff as a model, and stem cells are injected for repair. However, induced stem cells have not yet been used to repair degenerated rotator cuffs. Thus, we performed experiments to verify that the induced stem cells are better than un-induced stem cells for degenerated rotator cuff repair. We used a suitable concentration of 5-Aza to induce stem cells to repair the degenerated rotator cuff.

At present, the whole bone marrow adherence method, density gradient centrifugation, cell surface molecular marker sorting and cell screening are the methods that are mainly used for separation. In this study, primary BMSCS were obtained by density gradient centrifugation. To confirm that the obtained cells were in fact BMSCS, we detected the cell surface markers CD34, CD44 and CD90 by flow cytometry, and the analysis showed that the cells were positive for CD44 and CD90 and negative for CD34. Consistent with the literature [16-18] our extracted cells were mainly BMSCS and could thus be used for our experiments.

Cell viability evaluation using the MTT assay revealed that 5-Aza at concentrations of 5 and 10 μmol/L exerted no significant effect on cell viability, whereas 20 and 40 μmol/L 5-Aza significantly inhibited cell viability (P<0.05). These findings indicated that 20 and 40 μmol/L 5-Aza concentrations are toxic to cells and inhibit their viability.

Cell morphological changes in the experimental and control groups at different times were observed using an inverted light microscope. This analysis showed that group A exhibited no significant changes in cell morphology between days 1 and 9. The most obvious morphological changes were observed in group C. The results indicated that 5-Aza can initiate a mechanism that promotes BMSC differentiation and cell morphology changes. From days 1 to 5, no significant increase in cell number was observed in groups D and E, which indicates that 5-Aza at these concentrations, inhibits BMSC proliferation. On day 9, because the number of cells was too small, it was difficult to prepare cell slides and extract sufficient RNA for further testing. Therefore, groups D and E were not included in the subsequent analyses.

Actin is a component of the contractile apparatus and is regarded as a critical marker for muscle cell differentiation [19]. In this experiment, actin expression was observed in the control and experimental groups, but the expression levels in the different groups were different. Consistent with the results reported by Makino et al. [20] the semi-quantitative analysis of the fluorescence clearly showed that 10 μmol/L 5-Aza can significantly improve the myogenic differentiation of fifth-generation BMSCS, and the differences were statistically significant (p <0.05). This result indicated that 5-Aza exerts an effect on the differentiation of BMSCS into muscle cells and shows that 5-Aza inevitably initiates the partial differentiation of rabbit BMSCS into muscle cells.

Troponin is an important regulatory protein involved in the contraction of striated muscle and is present in the myocardium and skeletal muscle [21]. The troponin and myosin-related sequences in the extracted mRNA were amplified by RT-PCR, and different expression levels were obtained. The genetic-level analysis revealed that 10 μmol/L 5-Aza significantly (p <0.05) increased the mRNA levels of myosin and troponin, and thus, this concentration of 5-Aza can promote the myogenic differentiation of rabbit BMSCS.

5-Aza has been shown to be a common inducer of stem cell myogenesis [22,23]. In fact, it can induce the myogenic differentiation of stem cells mainly due to demethylation [10,11]. DNA methylation is fundamental for mammalian development, gene regulation, genomic imprinting, and chromatin structure [24]. Huri et al. [25] showed that the myogenesis efficiency of adipose stem cells can be increased by 5-Aza. Blaschke et al. [26] and Yin et al. [27] demonstrated changes in DNA methylation in response to specific media formulations and their nutritional factors. 5-Aza might demethylate genes related to BMSCS and promote the expression of genes related to myogenic differentiation, which would initiate differentiation into myocytes. Thus, 5-Aza plays a clear role in inducing the differentiation of BMSCS into myocytes *in vitro*. During this induction, the regulation of gene expression by demethylation and muscle differentiation are mainly responsible for the ability of BMSCS to differentiate into myocytes. Further studies of the demethylation effects induced by 5-Aza will provide a better understanding of the mechanisms through which the microenvironment induces BMSC differentiation.

Muscle tissue damage is a common disease, and BMSCS have been used to repair damaged skeletal tissue [28,29]. Transplanting the appropriate concentrations of 5-Aza-induced BMSCS into damaged skeletal muscle is a promising approach for repairing damage in the future. Because our concentration gradient used only 4 groups, it is impossible to fully explain the optimal concentrations of 10 μmol/L 5-Aza. However, our experiments have important significance for the

myogenic differentiation of rabbit BMSCS induced by 5-Aza in a rabbit model.

Conclusions

BMSCS are an ideal candidate for therapeutic applications because they are relatively easy to harvest, easily expandable *in vitro*, and can be isolated from adult bone marrow while retaining their differentiation potential. In our experiments, some BMSCS showed myogenic differentiation ability, but the myogenic differentiation rate was higher under 5-Aza induction. These findings support the new idea of inducing BMSCS with 5-Aza and then transplanting them into damaged skeletal muscles. This may be a better method for repairing skeletal muscle; however, these studies, although very promising, still need to be further expanded.

Declarations

Ethics approval and consent to participate, this study was approved by ethics of committee of Northern Jiangsu People's Hospital, the approval number is 2017040.

Competing Interest

The authors declare that they have no competing interests.

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Authors Contributions

Wenyong Fei, Yao Zhang and Jingcheng Wang: Conception and design, financial support, experiment, manuscript writing, final approval of manuscript, Yao Zhang made the equal contribution to the article and should be considered co-first author. Correspondence: Jingcheng Wang. Shichao Cao, Mingsheng Liu, Jiyang Tan, Xuanqi Wang, Bin Xie: experiment, analysis and interpretation of data and drafted the manuscript. All authors read and approved the final manuscript.

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