



6-Bromoindirubin-3'-oxime promotes osteogenic differentiation of canine BMSCs through inhibition of GSK3 β activity and activation of the Wnt/ β -catenin signaling pathway

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Abstract: This study aimed to investigate how 6-bromoindirubin-3'-oxime (BIO) increases the osteogenic differentiation of canine bone mesenchymal stem cells (BMSCs) and the role of the Wnt/ β -catenin signaling pathway in this process. We mimicked the effect of Wnt by adding BIO to the culture medium of BMSCs and examined whether canonical Wnt signaling positively affects the differentiation of these cells into osteoblasts. Canine BMSCs were cultured with 0.5 and 1.0 μ M BIO under osteogenic conditions and then differentiation markers were investigated. It was found that BIO significantly increased the activity of alkaline phosphatase (ALP), the number of ALP-positive cells, the mineralization level and calcium deposits. Moreover, cells cultured with 0.5 and 1.0 μ M BIO exhibited detectable β -catenin expression in their nuclei, and showed upregulated β -catenin and glycogen synthase kinase 3 beta (GSK3 β) phosphorylation compared to untreated cells. In addition, BIO enhanced the mRNA expression of osteoblast differentiation markers such as ALP, runt-related transcription factor 2, collagen I, osteocalcin, and osteonectin. In conclusion, BIO upregulated GSK3 β phosphorylation and inhibited its activity, thereby activating the Wnt/ β -catenin signaling pathway and promoting the osteogenic differentiation of canine BMSCs. The effect of 1.0 μ M BIO on BMSCs differentiation was stronger than that of 0.5 μ M BIO.

Key words: Bone mesenchymal stem cells, bromoindirubin oxime, osteogenetic differentiation, canine.

INTRODUCTION

Bone marrow mesenchymal stem cells (BMSCs) are non-hematopoietic pluripotent stem cells derived from the bone marrow that are capable of differentiating into a wide variety of cells such as neuronal, cardiac, skeletal muscle, epithelial, and pancreatic islet cells (Schwartz et al. 2002, Zhao et al. 2016, Özdal-Kurt et al. 2016, Zhang

et al. 2017). Bone marrow mesenchymal stem cells have been widely applied as seed cells for bone tissue engineering (Augello and De-Bari 2010, Deschaseaux et al. 2010, Kilinc et al. 2014, Liang et al. 2016, Duan et al. 2017). However, the ability of BMSCs to differentiate into osteoblasts is weak, which limits their clinical application in the treatment of orthopedic diseases. Thus, the key aim of the present study was to increase the differentiation ability of BMSCs. In veterinary clinics, pet fractures arising from bone disease,

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malnutrition, harmful (non-benign) arthropathy, and liver/kidney failure have gradually become serious obstacles affecting the quality of life and survival of pets, especially dogs and cats. Consequently, veterinary medical researchers and doctors are providing increasing attention to research around causes and possible treatments of pet fractures and/or bone diseases. However, conventional treatments of many bone-associated diseases, especially necrotic bone diseases, are characterized by long duration and unsatisfactory treatment outcomes, as they are mainly based on surgical debridement and fixation, and self-recovery following anti-inflammatory treatment. In order to improve the treatment outcome of these diseases, new methods capable of inducing the differentiation of BMSCs into osteoblasts are required. Therefore, a good understanding of the differentiation mechanisms of BMSCs is of great importance for the treatment of pet bone diseases. Since BMSCs are the source of osteoblast precursors (Pham et al. 2011, Park et al. 2010, Zhu et al. 2016), their differentiation *in vivo* is strictly controlled in terms of time and direction by an extremely complex set of signaling molecules and pathways, such as the Wnt, transforming growth factor beta, and bone morphogenetic protein signaling pathways (Augello and De-Bari 2010, Müller-Deubert et al. 2017, Long et al. 2017). Previous research has shown that the Wnt signaling pathway plays an important role in the differentiation, proliferation, and migration of osteoblasts (Ling et al. 2009, Robinson et al. 2006, Zhu et al. 2016, Zhou et al. 2016). Activation of the Wnt/ β -catenin signaling pathway stimulates osteogenic differentiation (Bennett et al. 2007, Su et al. 2015). In addition, the enhancement of Wnt signaling either by overexpression of Wnt (Bennett et al. 2007) or shortage of Wnt antagonists (Morvan et al. 2006, ten Dijke et al. 2008) increases bone formation in humans and mice. Moreover, Wnt signaling promotes the differentiation of mouse mesenchymal stem cells

(MSCs) towards the osteoblastic lineage (Gaur et al. 2005). 6-Bromoindirubin-3'-oxime (BIO), a specific inhibitor of glycogen synthase kinase 3 beta (GSK3 β), is known to activate the Wnt/ β -catenin signaling pathway (Meijer et al. 2003, Sato et al. 2004). In this study, we mimicked the effect of Wnt by adding BIO to canine BMSCs and investigated whether canonical Wnt signaling positively affects the differentiation of these cells into osteoblasts.

MATERIALS AND METHODS

MATERIALS

Chemicals were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise specified.

BMSCs ISOLATION AND CULTURE

The current protocol for experimentation on beagle dogs (two-months-old, 1.5 kg, obtained from Wu Gong County) was approved by the Institutional Animal Care and Use Committee of Northwest A&F University. Dogs were injected with 10 mg/kg ketamine. Then, 10 ml of the bone marrow was extracted from the iliac crest of each dog under sterile conditions according to previously reported methods (Sun et al. 2010). To obtain BMSCs, bone marrow cells were incubated in culture dishes (diameter, 60 mm) with 4 mL of alpha minimum essential medium (α -MEM, Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 100 U/mL penicillin/streptomycin (North China Pharmaceutical Co., Ltd., Shijiazhuang, China). Cultures were maintained at 37 °C and 5% CO₂. After 24 h, non-adherent cells were removed by replacing the medium with fresh α -MEM containing 10% FBS. The medium was replaced with fresh medium every 3 d. When cells reached 80-90% confluence, the medium was discarded, and 0.5 ml of 0.1% trypsin (Sigma) was added for 2 min at room temperature. Trypsin was neutralized by the addition of α -MEM containing 10% FBS, and the

cell suspension was centrifuged at $1000 \times g$ for 6 min. Then, the supernatant was discarded, and the cells were resuspended and passaged at 1:3 ratio.

IDENTIFICATION OF CELL SURFACE ANTIGENS BY FLOW CYTOMETRY

After the third passage BMSCs (P3 BMSCs) were harvested and fixed with 4% paraformaldehyde solution until further processing. For identification of cell surface antigens, cells were washed twice with phosphate-buffered saline (PBS) and directly labeled with fluorescein isothiocyanate (FITC)-conjugated CD34 (FITC Rat anti-Mouse CD34; BD Pharmingen), CD45 (FITC Rat Anti-Mouse CD45; BD Pharmingen), CD90 (PE Mouse Anti-Human CD90; BD Pharmingen), and CD105 (PerCP-CyTM5.5 Mouse anti-Human CD105; BD Pharmingen). Flow cytometry was performed using a FACS Calibur (BD Biosciences, San Diego, USA) and forward and side scatter profiles were adjusted to gate out debris and dead cells. Data were analyzed using Cell Quest software (BD Biosciences).

DETERMINATION OF SUITABLE BIO CONCENTRATION

To determine the BIO concentration needed to stimulate osteogenic differentiation of canine BMSCs, P3 BMSCs were cultured with different concentrations of BIO, and its effect on the cells was analyzed by cell proliferation detection and JC-1 assay.

Cell proliferation detection: P3 BMSCs were seeded at a density of 5.0×10^4 cells/mL into 24-well plate (400 μ L/well) in osteogenic medium containing (control group), and osteogenic medium containing 0.5, 1.0, and 2.5 μ M BIO. After 4 d, cells were counted using a red blood cell count plate (Qiujing, China) and proliferation was expressed as the number of population doublings per day (PDD). Average PDD values for each BIO concentration were calculated and results were

analyzed by paired Student's t-test relative to cells grown in basic medium without BIO.

JC-1 assay: P3 BMSCs were treated as described above. After 4 d, a JC-1 apoptosis detection kit (Keygen) was used to measure the mitochondrial transmembrane potential ($\Delta\psi_m$). After washing, the JC-1 working solution was added to each well, and cells were incubated at 37 °C and 5% CO₂ for 20 min, then the nuclei were stained with Hoechst33342 (Sigma, USA) for 5 min at room temperature. After washing with PBS, the images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

ALKALINE PHOSPHATASE STAINING

P3 BMSCs were seeded at a density of 5×10^4 cells/mL into 35-mm dishes (1 mL/ dish) with osteogenic medium containing different concentrations (0, 0.5 and 1.0 μ M) of BIO for 5 d. Alkaline phosphatase (ALP) activity was determined using an ALP kit (Nanjing Jiancheng Bioengineering Institute). The cells were washed 3 times with PBS, then fixed with 4% formaldehyde (Sigma, USA) for 30 min and washed with PBS again. Cells were stained with the ALP reagent [33 μ L BCIP (300 \times) and 66 μ L NBT (150 \times) were added to the alkaline phosphatase staining buffer] for 1-2 h at room temperature, then images were captured using an inverted microscope (Olympus, Tokyo, Japan), and the number of positive cells was obtained by randomly counting the number of positive cells in 3 fields of vision, repeating 3 times and averaging.

ALIZARIN RED STAINING

P3 BMSCs were seeded at a density of 1×10^4 cells/mL into 35-mm dishes (1 mL/ dish) for each group. After attachment, cells were cultured with an osteogenesis-inducing medium with or without BIO. On day 14, the cells were washed 3 times with PBS, fixed with 95% ethanol for 10 min, dried at room temperature, and then stained with 0.1%

Alizarin Red (pH 4.2; Sigma, USA) for 1-2 h at room temperature. Then, the Alizarin Red solution was removed, and cells were rinsed with PBS and dried at room temperature. Images were captured using an inverted microscope (Olympus, Tokyo, Japan).

IMMUNOFLUORESCENCE STAINING

P3 BMSCs were seeded at a density of 5×10^4 cells/mL into 35-mm dishes (1 mL/ dish) with osteogenic medium containing different concentrations (0, 0.5 and 1 μ M) of BIO for 4 d. Then, β -catenin was detected by immunofluorescence (IF) staining as follows: After discarding the culture medium, cells were washed 3 times with PBS and fixed with 4% paraformaldehyde (Sigma) for 1 h at room temperature, then washed 3 times with PBS again. The cells were dialyzed against 0.2% triton X-100 (Sigma, USA) for 10 min at room temperature, then washed 3 times with PBS and blocked with 1% bovine serum albumin (BSA, Sigma) in PBS at 4 °C for 1 h. After washing and draining, the cells were incubated overnight at 4 °C with the β -catenin primary antibody (1:100, Abcam, UK), washed 3 times with PBS, incubated with secondary donkey anti-rabbit antibody (1:100, Santa Cruz, USA) at 37 °C for 1 h, and then washed 3 times with PBS again. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, USA) for 2 min at room temperature. After washing with PBS, the images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

WESTERN BLOT ANALYSIS

P3 BMSCs were seeded at a density of 5×10^4 cells/mL into 35-mm dishes (1 mL/ dish) with osteogenic medium containing different concentrations (0, 0.5 and 1 μ M) of BIO. After 6 h, GSK3 β and phosphorylated GSK3 β (p-GSK3 β) were detected by western blotting, while β -catenin was detected after 4 d, also by western blotting. Cells were

lysed with radioimmunoprecipitation assay buffer (CW Bio, China) and protein concentrations were determined using the BCA Protein Assay Kit (Heart, China). Cell lysate was added to the loading buffer (CW Bio, China), boiled for 10 min, and then resolved on 10% sodium dodecyl sulfate-polyacrylamide gel. After electrophoresis, the gel was transferred to polyvinylidene difluoride membrane and blocked with 3% BSA (MP Biomedicals, France) in TBST buffer (0.1% (v/v) Tween-20 in TBS) for at least 2 h at room temperature. The following primary antibodies were used: anti- β -actin (No. 4970, Cell Signaling Technology; 1:4000), anti- β -catenin (ab32572, abcam; 1:2000), anti-GSK3 β (ab32391, abcam; 1:2000), and anti-p-GSK3 β (ab75814, abcam; 1:2000). Blots were incubated overnight with primary antibody in TBST at 4 °C. After rinsing, the membrane was incubated at room temperature with horseradish peroxidase-conjugated secondary antibody (Yeasen Bio, China) for at least 2 h. After washing for 3 times, the blots were visualized by enhanced chemiluminescence (Bio-Rad, USA).

REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION ANALYSIS

P3 BMSCs were seeded at a density of 1×10^4 cells/mL into 35-mm dishes (1 mL/ dish) for each group. After attachment, cells were cultured with an osteogenesis-inducing medium with or without BIO. After culture for different days, total RNA was extracted from the cells using TRIzol reagent (TaKaRa) according to the manufacturer's instructions, and quantified by spectrophotometry. Then, 2.0 μ g of total RNA was treated with gDNA Eraser Buffer and gDNA Eraser to remove genomic DNA traces that might interfere with the polymerase chain reaction (PCR). First strand cDNA synthesis was carried out using the PrimeScript RT Enzyme Mix I (TaKaRa) with RT Primer Mix and 5X PrimeScript Buffer 2 at 37 °C for 15 min, then at 85 °C for 5 s. From the prepared cDNA, only 1 μ L

was used as a template for the reverse transcription-PCR (RT-PCR) with osteoblast-specific gene primers for *ALP*, runt-related transcription factor 2 (*RUNX2*), collagen I (*COI*), osteocalcin (*OCN*), and osteonectin (*ONN*). *GAPDH* was used as the internal reference gene for the osteoblast-specific genes. Primers for osteoblast-specific genes are listed in Table I. Primers were synthesized by Invitrogen. Polymerase chain reactions were run in a total volume of 20 μL , made by adding 10 μL SYBR Premix Ex Taq II to a mixture containing 0.4 μL ROX Reference Dye II, RNase-free dH_2O and 0.8 pmol of each primer. Reaction mixtures were incubated at 95 $^\circ\text{C}$ for 30 s, followed by 40 cycles at 95 $^\circ\text{C}$ for 5 s and at the specific annealing temperature (Table I) for 30 s. In all cases, each PCR was performed with triplicate samples and repeated on at least 3 independent samples on an Applied Biosystems thermocycler (Thermo Fisher Scientific, USA). The fold change in the expression of each gene was calculated by the $2^{-\Delta\Delta\text{Ct}}$ method.

All collected data were entered in a Microsoft Excel sheet after generation of a proper template, and were calculated and analyzed by SPSS software (version 18.0) (International Business Machines (IBM) Corporation, New York, USA).

RESULTS

PHENOTYPE CHARACTERIZATION

Canine BMSCs were analyzed for their cell surface antigens by flow cytometry (Figure 1). Our results showed that isolated BMSCs were strongly positive for CD105 and CD90, which are specific antigens for non-hematopoietic bone marrow progenitor cells, and were negative for CD34 and CD45, which are common markers of hematopoietic and endothelial cells (Tuli et al. 2003). These results demonstrate the immunophenotypic MSC characteristics of canine BMSCs.

EFFECT OF BIO ON BMSCs PROLIFERATION

As shown in Figure 2, treatment of canine BMSCs with 0.5 and 1.0 μM BIO slightly inhibited their proliferation (Figure 2a, b). The antiproliferative activity of BIO was dramatically enhanced at the concentration of 2.5 μM , as cells treated with 2.5 μM BIO showed slow proliferation and demonstrated significant morphological changes; most of them became irregularly circular and hypertrophic (Figure 2a, b). Even though a small fraction of the cells still maintained their fibroblast morphology on day 4, their shapes changed and became shorter and thicker. The control group demonstrated normal fibroblast morphology on day 4 (Figure 2b).

The $\Delta\psi\text{m}$ of the BMSCs was determined by JC-1 staining (Figure 2c). Strong red and weak green fluorescent signals were detected in osteogenic media containing 0, 0.5, and 1.0 μM BIO, while the osteogenic medium containing 2.5 μM BIO demonstrated relatively strong green and relatively weak red fluorescent signals. The significant decrease in the fluorescence intensity of the red signals in the 2.5 μM BIO group compared to the other groups indicates a decrease in the $\Delta\psi\text{m}$ in this group. Based on the results of the cells proliferation and JC-1 staining assays, 0.5 and 1.0 μM were selected as the optimal BIO concentrations.

BIO ENHANCED THE DIFFERENTIATION OF CANINE BMSCs INTO OSTEOBLASTS

The incubation of canine BMSCs with various concentrations of BIO for 5 d resulted in an increase in the activity of ALP (Figure 3a, b), which is a marker enzyme for osteoblasts. As shown in Figure 3, treatment with 0.5 and 1.0 μM BIO significantly increased the number of ALP-positive BMSCs (Figure 3a, b), with 1.0 μM BIO exhibiting a stronger effect than 0.5 μM BIO. Moreover, treatment with 0.5 and 1.0 μM BIO for 14 d resulted in an increase in the mineralization level

TABLE I
Primers used in the reverse transcription-quantitative polymerase chain reaction analysis.

Gene	Genbank NO	Primer sequence (5'-3')	Annealing temperature (°C)	Product size (bp)
<i>RUNX2</i>	XM_011514964	F:TGCCTCTGGCCTTCCACTCTCAG R:TGCATTTCGTGGGTTGGAGAAGCG	60	131
<i>ALP</i>	XM_005617212	F:CCAAGCTCAACAGACCCTGA R:GAGACACCCATCCCATCTCC	60	102
<i>COL</i>	NM_001003187	F:CTATCAATGGTGGTACCCAGTTT R:TGTTTTGAGAGGCATGGTTG	60	111
<i>OCN</i>	XM_014115322	F:ATGCGGTCCCTGATGGTC G:AGCCAGAGTCCAGGTAGC	60	179
<i>ONN</i>	XM_014113053	F:GAAGGTGCTGAGGAAACTGAAGAGG R:GAAGTGGCAGGAAGAGTCAAAGGT	60	207
<i>GAPDH</i>	NM_001003142	F:ATCACTGCCACCCAGAAGAC R:TGTAGCCCAGGATGCCTTTG	60	289

F: forward; R: reverse. Marker genes of osteogenic cell - *RUNX2*: Runt-related transcription factor 2; *ALP*: alkaline phosphatase; *COL*: collagen I; *OCN*: osteocalcin; *ONN*: osteonectin; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

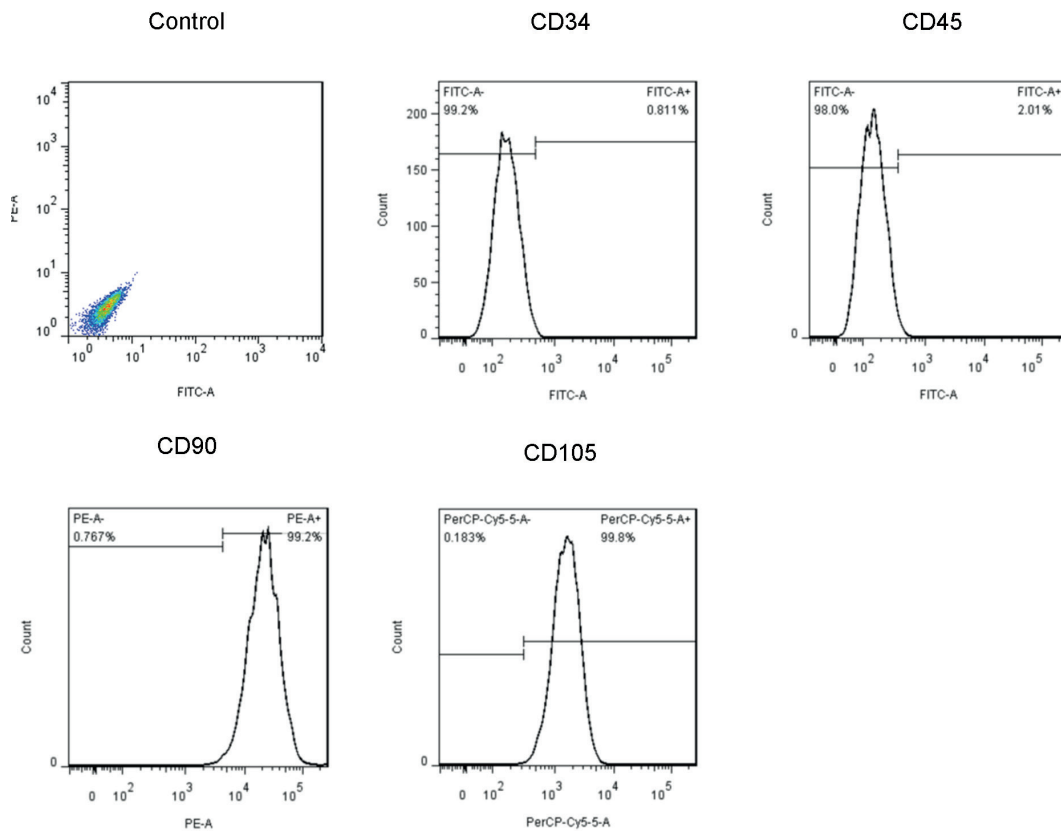


Figure 1 - Detection of cell surface antigen of canine BMSCs by Flow cytometry. (n = 3). Canine BMSCs highly expressed CD105 (99.8%) and CD90 (99.2%), and lowly expressed CD45 (2.01%) and CD34 (0.811%).

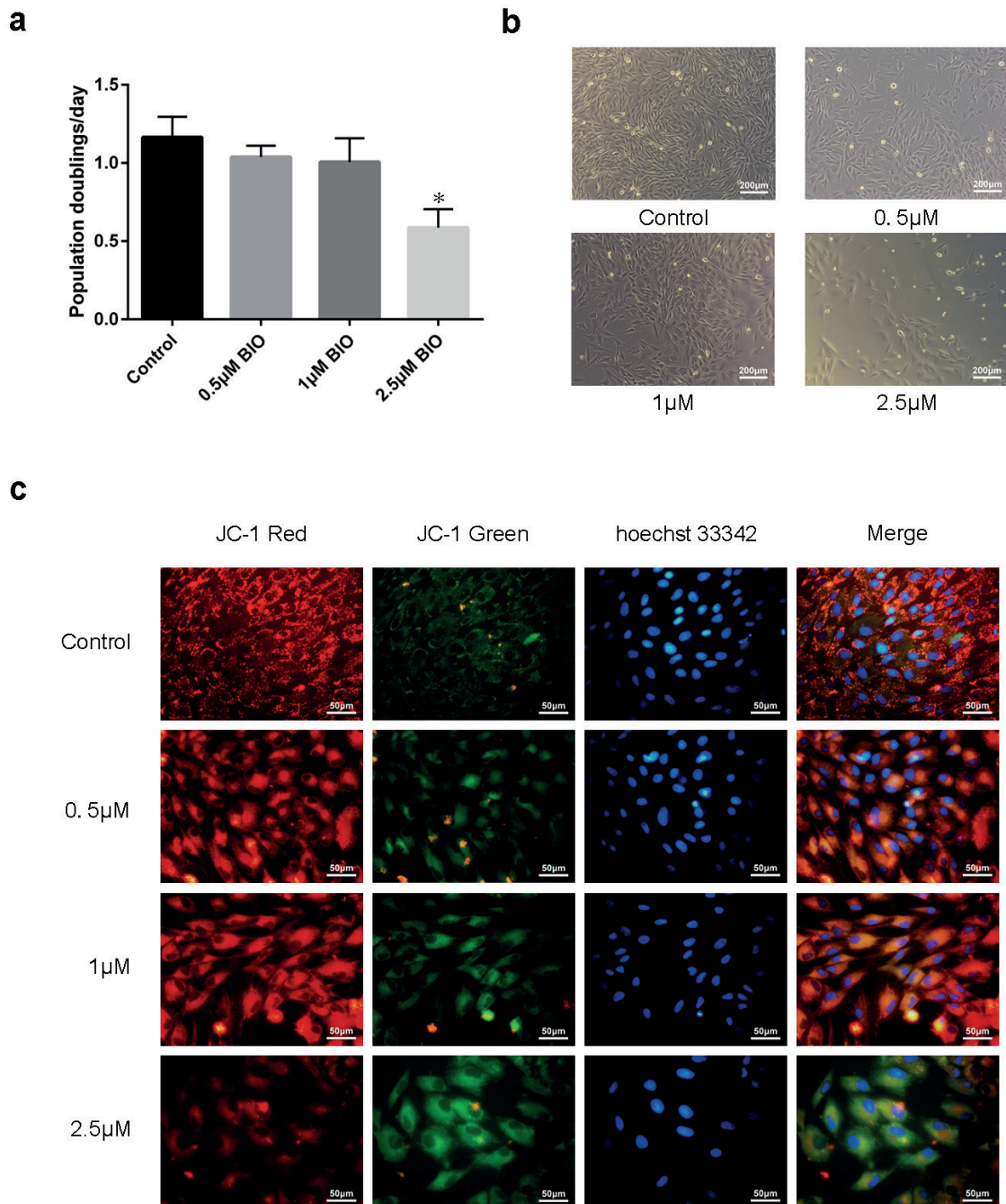


Figure 2 - Effect of BIO on morphology, proliferation and mitochondrial transmembrane potential of canine BMSCs. Control: osteogenic medium; 0.5, 1.0 and 2.5µM BIO: osteogenic medium containing 0.5, 1.0 and 2.5 µM BIO. (a) Effect of BIO on proliferation of canine BMSCs. (n = 3). Error bars denote mean ± SD. *P < 0.05. (b) The morphology and proliferation of canine BMSCs grown for 4 days different medium as indicated. (c) Changes in the mitochondrial transmembrane potential of canine BMSCs grown for 4 days different medium as indicated. The red fluorescence (polymer) and green fluorescence (monomer) of canine BMSCs in osteogenic medium with or without BIO. Normally, mitochondria JC-1 use its potential as a red fluorescent emitter to form a polymer. In the destruction of mitochondrial function, JC-1 as a monomer dispersed and distributed in the cytoplasm, was detected as green fluorescence. Hoechst33342: Hoechst33342 staining of the nucleus; Merge: The superposition of red fluorescence (polymer), green fluorescence (monomer) and Hoechst33342 in canine BMSCs.

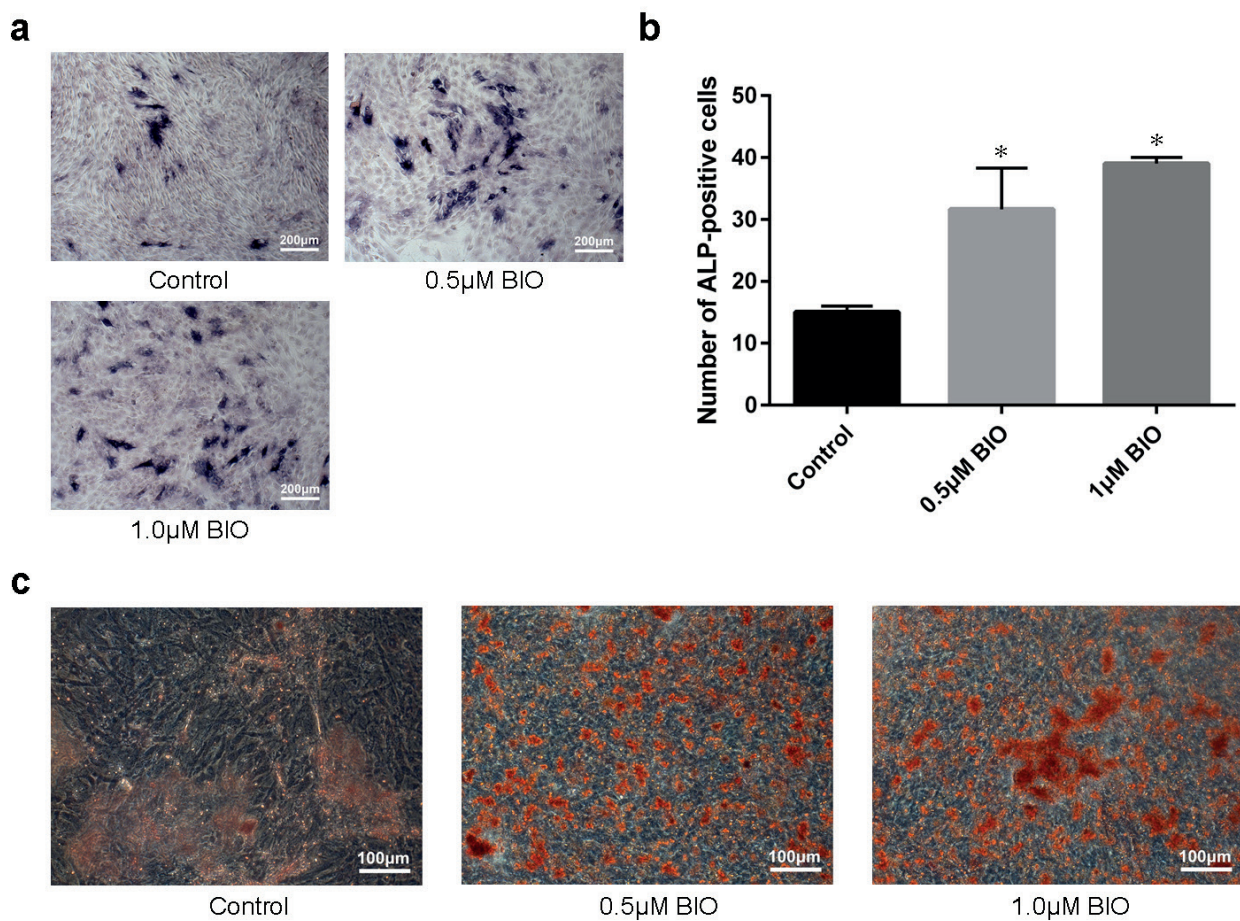


Figure 3 - BIO increased canine BMSCs differentiation towards osteoblasts. (a) BIO enhances the number of ALP staining-positive cells and ALP activity. (b) Number of ALP-positive cells. Canine BMSCs were incubated with osteogenic medium with or without BIO for 5 days, and fixed for ALP staining or quantitative assay of ALP activity by using p-Npp as a substrate. (n = 3). Error bars denote mean ± SD. *P < 0.05. (c) BIO enhanced bone mineralization in canine BMSCs. Canine BMSCs were incubated with osteogenic medium followed by addition of 0, 0.5 and 1.0 µM BIO for 14 d and fixed for alizarin red staining.

and calcium deposits, as revealed by Alizarin Red staining (Figure 3c). All these results suggest that BIO treatment could facilitate the differentiation of BMSCs into osteoblasts.

BIO INHIBITED GSK3B ACTIVITY AND PROMOTED THE ACCUMULATION OF β -CATENIN IN THE NUCLEUS OF CANINE BMSCs

β -Catenin is known to be an important transcriptional regulator in the Wnt signaling pathway; the nuclear accumulation of β -catenin is a critical step in Wnt/ β -catenin signaling. Being a specific inhibitor of GSK3 β , BIO can promote the accumulation of β -catenin in the nucleus and thus activate Wnt/ β -

catenin signaling. Results of western blotting and β -catenin IF staining demonstrated that BIO activated the Wnt/ β -catenin signaling pathway in canine BMSCs (Figure 4). Treatment of the cells with 0.5 and 1.0 µM BIO for 4 d resulted in a significant upregulation in β -catenin levels, with 1.0 µM BIO showing a stronger effect than 0.5 µM BIO (Figure 4a). Moreover, cells treated with 0.5 and 1.0 µM BIO demonstrated detectable β -catenin expression in the nuclei compared to control cells (Figure 4b). Furthermore, we examined the direct inhibitory activity of BIO on GSK3 β . Canine BMSCs were cultured with 0.5 and 1.0 µM

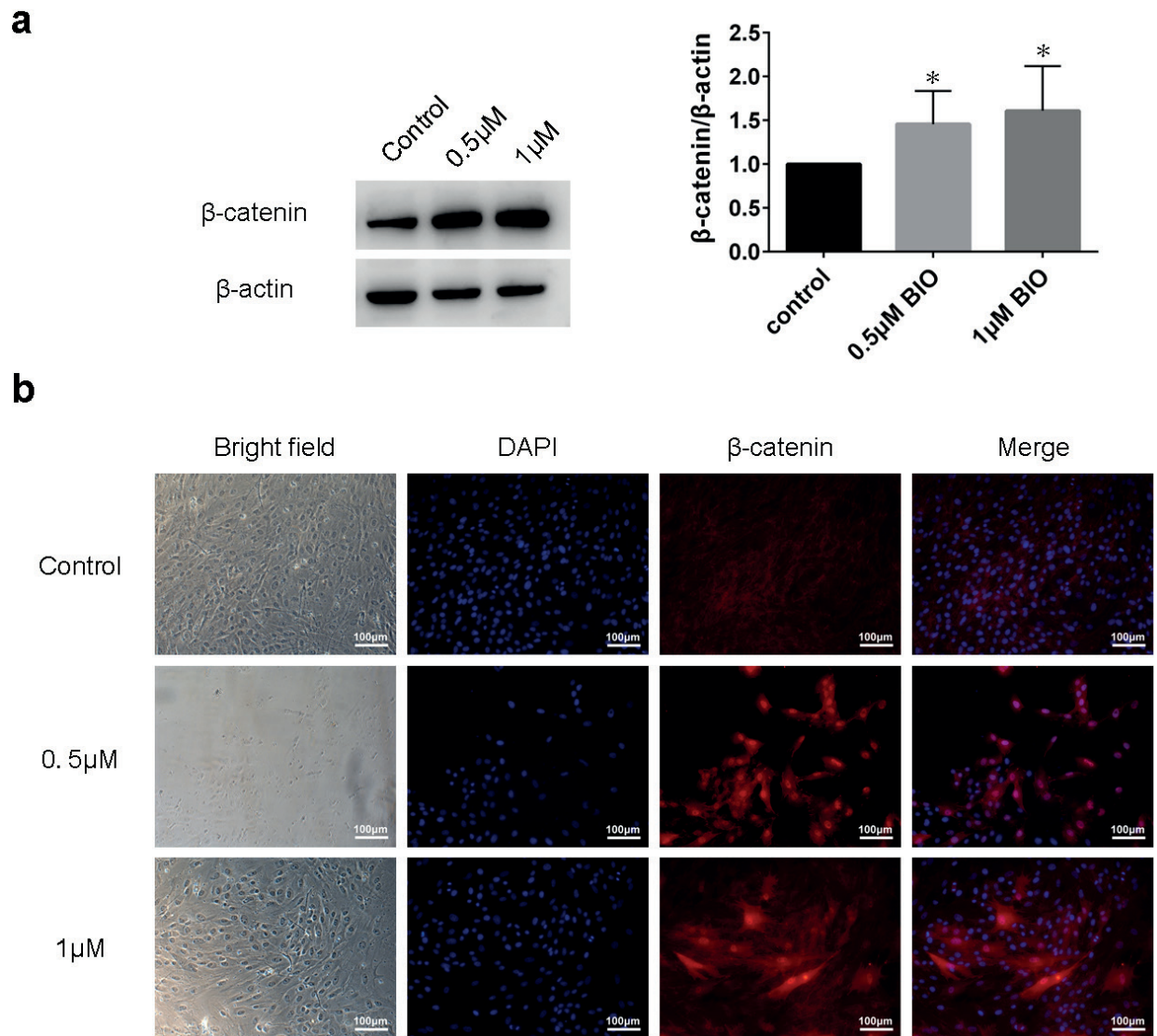


Figure 4 - BIO activated Wnt/ β -catenin signalling pathways in canine BMSCs. (a) BIO up-regulated β -catenin expression in canine BMSCs. (b) BIO promoted the accumulation of β -catenin in the cell nuclei. Canine BMSCs were incubated with basic medium, osteogenic medium with or without BIO for 4 days, detected expression of β -catenin by Western blot and immunocytochemistry staining. (n = 3). Error bars denote mean \pm SD. *P < 0.05. Control: osteogenic medium; 0.5 and 1.0 μ M BIO: osteogenic medium containing 0.5 and 1.0 μ M BIO. DAPI: DAPI staining of the nucleus. β -catenin: β -catenin immunocytochemistry staining in nucleus. Merge: the superposition of DAPI and β -catenin immunocytochemistry in canine BMSCs.

BIO under osteogenic conditions for 6 h. Then, GSK3 β and p-GSK3 β levels were determined by western blotting. Our results showed that GSK3 β phosphorylation was significantly upregulated by BIO treatment, and that p-GSK3 β levels in cells treated with 1.0 μ M BIO were slightly higher than in cells treated with 0.5 μ M BIO (Figure 5). These results indicate that BIO inhibits GSK3 β activity,

suggesting that GSK3 β is the target of BIO for enhancing osteogenesis.

BIO INCREASED THE MRNA EXPRESSION OF *RUNX2*, *ALP*, *COL*, *ONN*, AND *OCN*.

To investigate the effect of BIO on osteoblast differentiation, canine BMSCs were incubated with 0, 0.5, and 1.0 μ M BIO for 3, 7, and 14 d, and

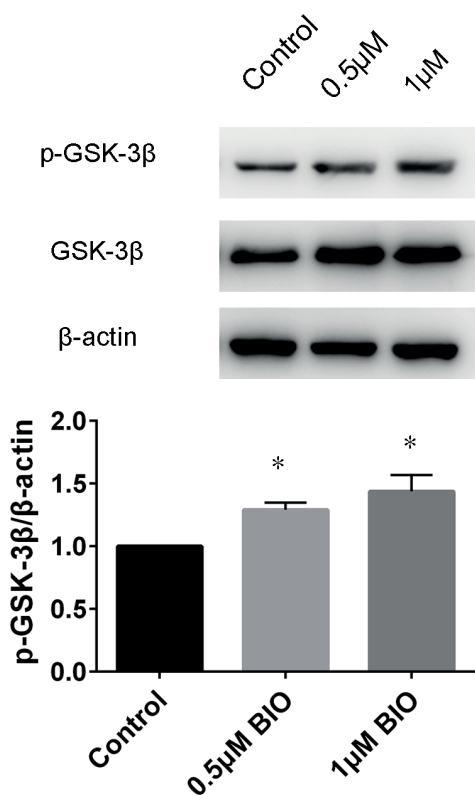


Figure 5 - BIO enhanced the phosphorylation of GSK3 β in canine BMSCs. Canine BMSCs were incubated with osteogenic medium with or without BIO for 6 hours, detected expression of phosphorylation of GSK3 β by Western blot. (n = 3). The Gel Pro Software (Tanon Science & Technology Co., Ltd., China) was used to determine the gray values of the internal reference (β -actin) proteins and p-GSK3 β protein bands, and the ratios were used as the relative expression of proteins. Calculating p-GSK3 β relative expression and averaging. Error bars denote mean \pm SD. *P < 0.05. Control: osteogenic medium; 0.5 and 1.0 μ M BIO: osteogenic medium containing 0.5 and 1.0 μ M BIO.

the expression levels of osteoblast differentiation markers, such as *RUNX2*, *ALP*, *COI*, *ONN*, and *OCN* were examined by RT-PCR. As shown in Figure 6, *COI* and *RUNX2* expression levels were evidently increased in cells treated with 0.5 and 1.0 μ M BIO for 3, 7, and 14 d. Moreover, the expression of *ALP*, a marker of early osteoblast differentiation, was evidently increased in cells treated with 0.5 and 1.0 μ M BIO for 3 and 7 d. However, cells cultured with BIO for 14 d showed lower relative mRNA expression of *ALP* than

control cells. Moreover, markers of late osteoblast differentiation, *OCN* and *ONN*, were found to be strongly expressed in BMSCs treated with 0.5 and 1.0 μ M BIO for 14 d. All these results indicate that BIO can promote the ability of canine BMSCs to differentiate into osteoblasts.

DISCUSSION

Previous research has shown that the Wnt signaling pathway plays an important role in the differentiation, proliferation, and migration of stem cells (Ling et al. 2009, Robinson et al. 2006, Zhu et al. 2016, Zhou et al. 2016), as Wnt signals play different roles under different conditions. For example, activation of the Wnt signaling pathway stimulates the differentiation of mouse MSCs towards the osteoblastic lineage (Gaur et al. 2005). In addition, activation of the Wnt/ β -catenin signaling pathway by inhibition of GSK3 β (Dravid et al. 2005, Anton et al. 2007) is important for the maintenance of pluripotency and self-renewal in human and mouse embryonic stem cells (Hur and Zhou 2010, Meijer et al. 2003), and for the modulation of apoptosis (Chon et al. 2015). In this study, we added different concentrations of BIO to BMSCs and examined whether the stimulation of canonical Wnt signaling by BIO positively affects the differentiation of these cells into osteoblasts. First, we found that 2.5 μ M BIO inhibited the proliferation of BMSCs, thus, lower concentrations (0.5 and 1 μ M BIO) were selected to be used in the experiments.

Osteogenic differentiation of BMSCs is the most important process in bone formation and reconstruction and is regulated by multiple signaling pathways (Lee et al. 2016, Zhou et al. 2016, Tang et al. 2016, Li et al. 2016). The classic Wnt/ β -catenin signaling pathway is one of the key pathways that regulate bone formation and promote bone differentiation (Qian et al. 2015, Ma et al. 2016, Liu et al. 2016). The Wnt/ β -catenin signaling pathway

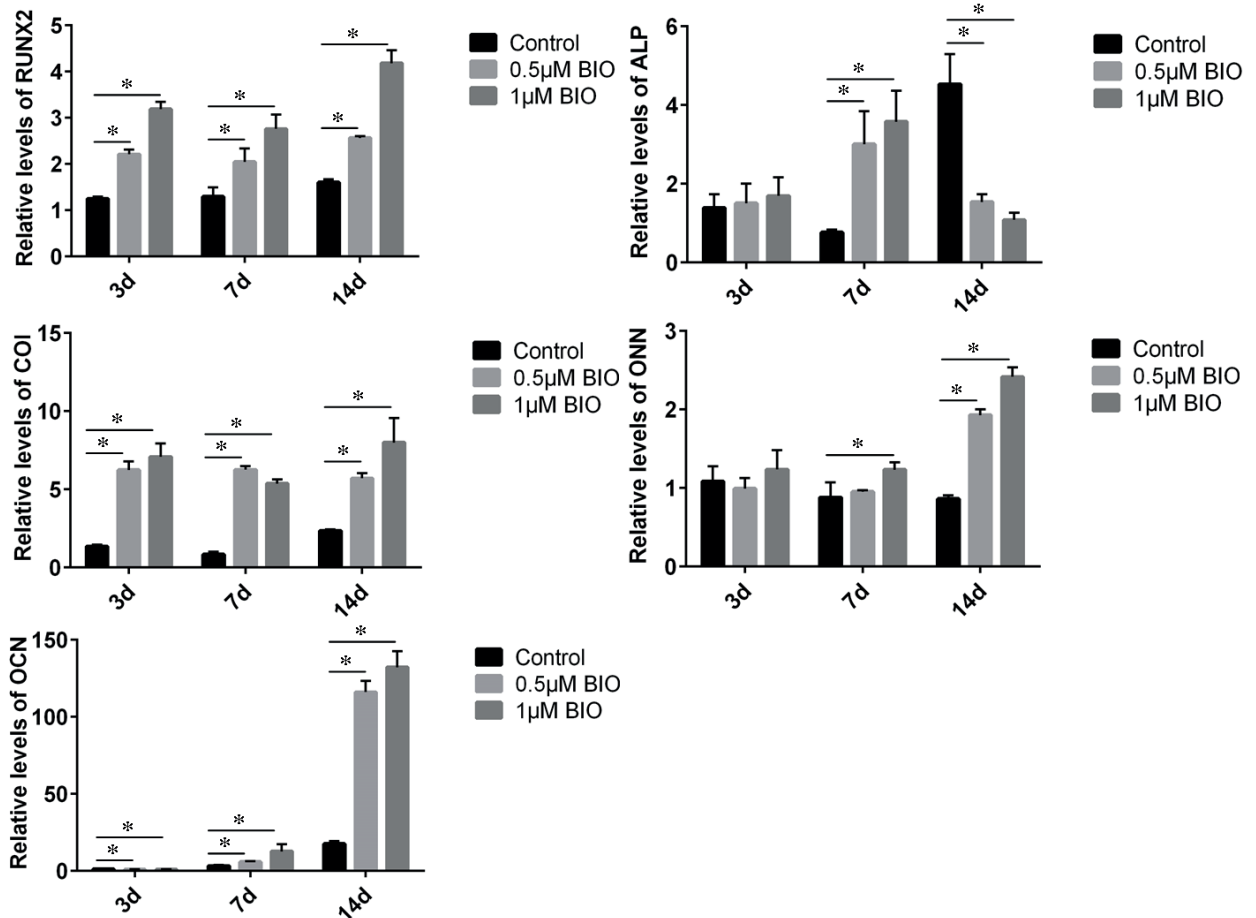


Figure 6 - BIO enhanced the mRNA expression of osteoblast differentiation markers in canine BMSCs. Canine BMSCs were incubated with osteogenic medium with or without BIO for 3 d, 7 d and 14 d, collected the cells and detected expression of *RUNX2*, *ALP*, *COI*, *ONN*, and *OCN* mRNA by qPT-PCR. (n = 3). Error bars denote mean ± SD. *P < 0.05. Control: osteogenic medium; 0.5 and 1.0 μM BIO: osteogenic medium containing 0.5 and 1.0 μM BIO.

functions by regulating β -catenin phosphorylation and degradation in the cytoplasm. Upon activation of the canonical Wnt pathway, inhibition of GSK3 β results in the dephosphorylation of β -catenin leading to its accumulation in the nucleus. In this study, we investigated the effects of BIO on the Wnt/ β -catenin signaling pathway, and on the differentiation of canine BMSCs. Our results demonstrated that BIO treatment resulted in β -catenin upregulation and nuclear translocation, which indicates that BIO could activate Wnt/ β -catenin signaling in canine BMSCs. Moreover, we found that BIO can robustly stimulate canine BMSCs differentiation; 0.5 and 1.0 μM BIO successfully enhanced the

differentiation of canine BMSCs into osteoblasts. Furthermore, we investigated the direct inhibitory effect of BIO on GSK3 β by western blot analysis, which demonstrated an upregulation in p-GSK3 β levels in BIO-treated cells, indicating that the BIO-induced activation of Wnt/ β -catenin signaling in canine BMSCs is mediated by inhibition of GSK3 β activity.

The effect of the Wnt pathway on osteogenesis depends on both the cellular environment and the expression of target genes (Liu et al. 2013). Thus, we investigated the expression of osteoblast differentiation markers such as *RUNX2*, *COI*, *ALP*, *ONN*, and *OCN*, and found that they were increased

in cells treated with BIO. *RUNX2* activates and induces the differentiation of BMSCs into immature osteoblasts and regulates pre-osteoblast maturation. It also directly stimulates the transcription of *OCN* and *COI* during the differentiation of BMSCs into osteoblasts (Ducy et al. 1999, Okazaki and Sandell 2004). *COI* is the basis for the formation of calcium nodules (Owen et al. 1990), *ALP* activity is a useful early detector for features of osteogenesis, and *OCN* is a late marker of osteogenic differentiation (Maroni et al. 2012). Our results showed that, compared with the control cells, BIO-treated cells exhibited an evident increase in the relative mRNA expression of *RUNX2* and *COI*. In addition, relative mRNA expression of *ALP* was evidently increased in cells treated with BIO for 3 and 7 d, while it was decreased in cells treated with BIO for 14 d. Moreover, *OCN* and *ONN*, markers of late osteoblast differentiation, were strongly expressed in BMSCs treated with BIO for 14 d compared to untreated cells, which indicates the formation of mature osteoblasts from canine BMSCs treated with BIO for 14 d. Alizarin Red staining proved osteoblast formation and matrix mineralization in BIO-treated cells on day 14, while it demonstrated their absence in the control cells. Collectively, all of the aforementioned results prove that BIO could increase the differentiation ability of BMSCs into osteoblasts through regulation of the target genes of β -catenin signaling.

CONCLUSIONS

Treatment of canine BMSCs with BIO resulted in upregulation of GSK3 β phosphorylation, and thus inhibition of GSK3 β activity and activation of Wnt/ β -catenin signaling, consequently leading to accumulation of β -catenin in the cytoplasm and its translocation into the nucleus, where it induced the expression of downstream target genes and osteoblast differentiation markers. In conclusion, BIO-mediated activation of the canonical Wnt/ β -

catenin signaling pathway can increase the ability of canine BMSCs to differentiate into osteoblasts.

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AUTHOR CONTRIBUTIONS

All authors contributed equally to this work. Baohua Ma designed the study, Xiao-e Zhao and Zhenshan Yang performed the experiments and prepared the manuscript. Zhen Gao and Junbang Ge quantified gene expression, Qiang Wei collected and analyzed data. All authors discussed the results and implications and commented on the manuscript at all stages.

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