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Original Article

Regulation of osteogenic differentiation of bone marrow mesenchymal stem cells by intermittent hypoxia

Silong Wei[1,2,3*], Xiaojing Guo[4*], Xiaofeng Lu[1,2,3]

[1]Department of Oral and Craniomaxillofacial Surgery, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai City, P.R. China; [2]Shanghai Key Laboratory of Stomatology and Shanghai Research Institute of Stomatology, Shanghai City, P.R. China; [3]National Clinical Research Center of Stomatology, Shanghai City, P.R. China; [4]Oral Biomedical Engineering Laboratory, Shanghai Stomatological Hospital, Fudan University, Shanghai City, P.R. China. [*]Equal contributors and co-first authors.

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Abstract: Objective: The aim of this study was to explore the effects of intermittent hypoxia on osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). Methods: Primary culturing of rat BMSCs was conducted by adherent cell culturing. BMSCs were identified with immunofluorescence staining. Third-generation BMSCs were randomly divided into the control group and intermittent hypoxia group. The two groups were compared in the formation of mineralized bone nodules after 3 weeks of culture and Real-time PCR was performed for testing the mRNA levels of the bone differentiation markers (ALP, Col-1 and BMP-2) in the two groups at 24 h, 48 h and 72 h, respectively. Results: BMSCs were successfully cultured in this trial. Significantly more mineralized nodules of BMSCs, at different time points, were observed in the intermittent hypoxia group than the control group (P<0.001). Moreover, mRNA levels of ALP, Col-1, and BMP-2 in the intermittent hypoxic group were significantly higher than those in the control group (all P<0.01). Conclusion: Intermittent hypoxia improves osteogenic differentiation of BMSCs.

Keywords: Intermittent hypoxia, bone marrow mesenchymal stem cells, osteogenic differentiation, obstructive sleep-apnea hypopnea syndrome

Introduction

Obstructive sleep apnea-hypopnea syndrome (OSAHS) is a clinically common condition [1, 2]. Recurrent apnea accompanied by intermittent hypoxemia, namely, periodic increase-decrease of oxygen saturation, is the most prominent characteristic of OSAHS. It is the basis for other organ injuries in the body [3, 4]. Recent studies investigating the pathophysiology of OSAHS have shown that OSAHS has a serial pathophysiological process that affects osseous development and metabolism [5, 6]. Other studies have reported elevated bone resorption markers in OSAHS populations [7, 8]. Another study stated that microscopic changes in the physiological state were related to osseous development and metabolism in OSAHS patients [9]. Intermittent hypoxia has been reported to stimulate the early mobilization of bone marrow mesenchymal stem cells (BMSCs) into the blood, playing roles in anti-inflammation and injury repair of vascular endothelial cells [10-12]. BMSCs are a source of osteoblasts. The biological processes of BMSCs, including proliferation and differentiation, are extremely important in bone injury repair, growth, remodeling, and regeneration [13]. Basic research concerning the effects of OSAHS on osseous development and metabolism has been rarely conducted. Therefore, in the present study, an intermittent hypoxic model was established with BMSCs as study subjects, aiming to observe the effects of intermittent hypoxia on proliferation and osteogenic differentiation of BMSCs, *in vitro*, expecting to gain a better understanding of bone healing capacities in OSAHS populations.

Materials and methods

Experimental animals

Twenty-five SPF healthy female SD rats, weighing about 200 g, were provided by the Center for Experimental Animals of the Ninth People's

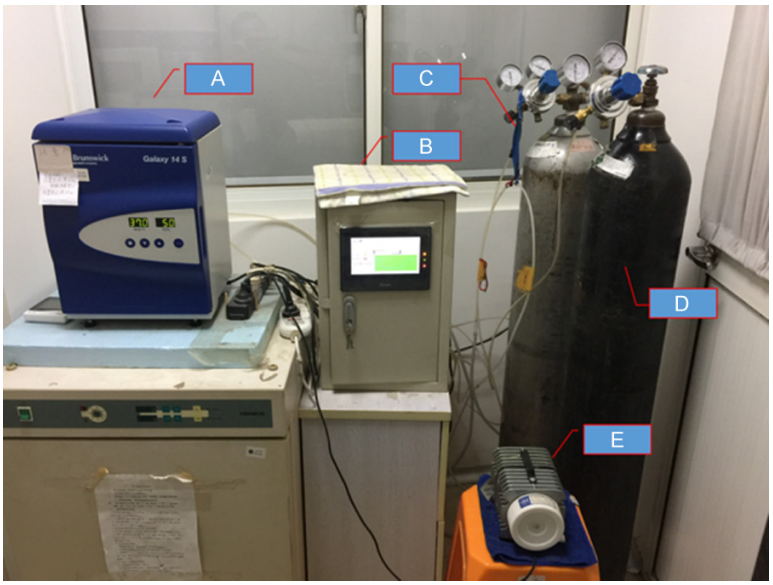

Figure 1. System for developing a cell model of intermittent hypoxia. A: Carbon dioxide cell incubator; B: Single chip microcomputer; C: Carbon dioxide; D: Nitrogen; E: Air compressor.

Hospital, Shanghai Jiao Tong University School of Medicine.

Establishment of an intermittent hypoxic cell model

An intermittent hypoxic cell model was established, according to methods reported by Gozal et al. [14]. The system for developing cell models of intermittent hypoxia was provided by the Laboratory of Department of Respiratory Medicine of Ruijin Hospital, Shanghai Jiaotong University (**Figure 1**). Modes of intermittent hypoxia were set to be hypoxia (5% CO_2, 1% O_2) for 3 minutes and 20 seconds and reoxygenation (5% CO_2, 20.5% O_2) for 3 minutes and 20 seconds, with hypoxia and reoxygenation performed alternately. Cells were assayed at 24 hours, 48 hours and 72 hours after intervention.

Culture and identification of rat BMSCs

SD rats were sacrificed by cervical dislocation, soaked in 75% alcohol, and transferred to an aseptic operating table. Here, the skin of the rats was cut open, fat, muscle, and other soft tissues were removed, the femur and tibia were dissected, the metaphysis was cut off, and the medullary cavity was rinsed with a sterile syringe containing DMEM culture medium (10 mL). Contents of the medullary cavity were rinsed into a centrifuge tube until the medullary cavity was white. Collected pellets were centrifuged for 5 minutes at 1000 r/min. The supernatant was removed and the precipitates were treated in 10 mL of a high-glucose DMEM medium containing 10% fetal bovine serum (FBS) to prepare bone marrow cell suspension. Cells were inoculated in a culture dish (10 cm) at a density of $4*10^5/cm^2$ and cultured in a cell incubator (37°C and 5% CO_2). Two days later, half of the culture medium was removed and an equal amount of new culture medium was added, with the solution replaced once every 3 days. When the cells reached 70-80% confluence, they were trypsinized and sub-cultured. Third-generation BMSCs were used in this experiment. Immunofluorescence was used for detection of surface antigens (CD44, CD90, CD105, CD34 and CD45) in rat BMSCs [15].

BMSCs osteogenic differentiation

The bottom of a 6-well plate was pre-coated with 0.1% gelatin. After standing for 30 minutes, gelatin was removed and the plate was used for inoculation of the third-generation BMSCs. When cells reached 70% confluence, the medium was supplemented with osteogenic induction medium for induction of osteogenic differentiation. Cells were randomly assigned to the intermittent hypoxia group and control group. The osteogenic induction medium was prepared by mixing basal medium (175 mL), FBS (20 mL), penicillin-streptomycin solution (2 mL), glutamine (2 mL), oascorbic acid (400 μL), sodium β-glycerophosphate (2 mL), and dexamethasone (2 μL). The culture medium was changed every 3 days. After 3 weeks of culturing, the osteogenic differentiation medium was aspirated from the 6-well plate. Cells were washed twice with PBS and fixated for 30 minutes after addition of 4% paraformaldehyde solution (2 mL) to each well. After paraformaldehyde solution was removed, the cells were rinsed twice with PBS and stained for 3 minutes after addition of the solution of alizarin red

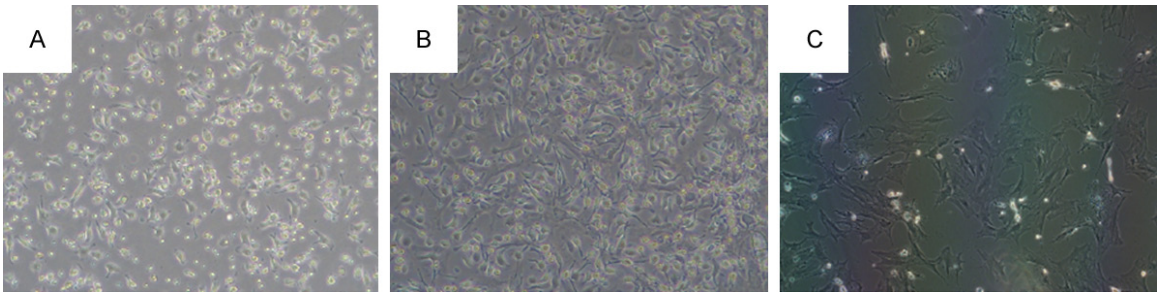

Figure 2. Culture of rat BMSCs (100×). A: Primary BMSCs cultured for 1 day; B: Primary BMSCs cultured for 8 days; C: Third-generation BMSCs.

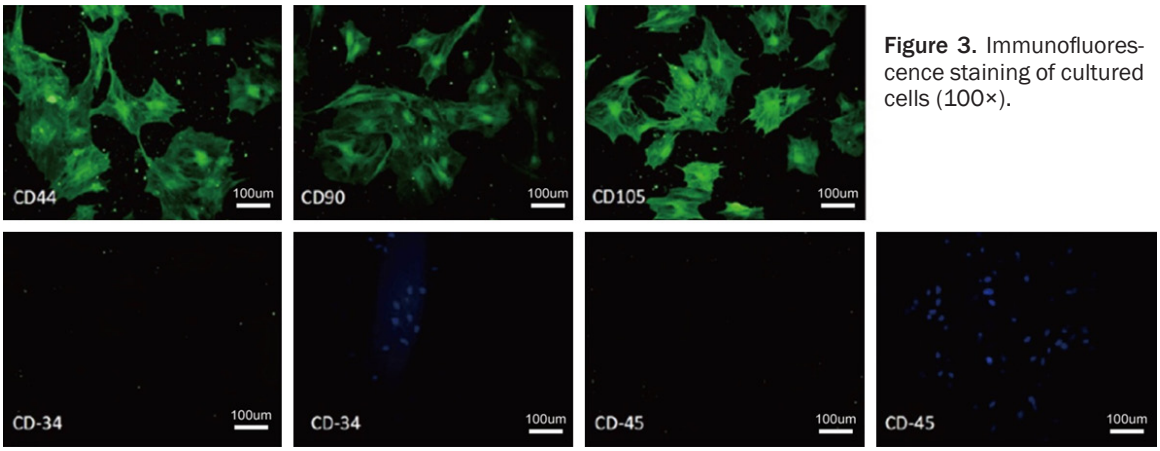

Figure 3. Immunofluorescence staining of cultured cells (100×).

Table 1. Number of mineralized nodules formed in two groups (n)

Variable	1 week	2 weeks	3 weeks
Control group	1.8±0.3	2.2±0.3	2.8±0.5
Intermittent hypoxic group	3.4±0.4	4.5±0.7	5.3±0.8
t	9.051	8.542	7.495
P	<0.001	<0.001	<0.001

(1 mL) to each well. The stain solution was removed and the cells were washed twice with PBS. Cells were photographed under an inverted microscope, with 8 fields of view randomly selected for per well. The number of mineralized nodules in the two groups was calculated. Five replicate wells were set for each group and averaged.

ALP, Col-1 and BMP-2 (osteogenic differentiation markers) levels detected by real-time PCR

Total RNA was extracted with TRIzol reagent from each group. cDNA was synthesized from total RNA by reverse transcriptase. The β-actin gene was utilized as an internal control. The forward primer of β-actin was 5'-CATTAAGGAGAAGCTGTGCT-3', and the reverse primer of β-actin 5'-GTTGAAGGTAGTTTCGTGGA-3'; the forward primer of Col-1 was 5'-GGTCCCAAAGGTGCTGATGG-3', and the reverse primer of Col-1 5'-GACCAGGCT CACCACGGTCT-3'; the forward primer of ALP was 5'-GTCCCACAAGAGCCCACAAT-3', and the reverse primer 5'-CAACGGCAGAGCCAGGAAT-3'; the forward primer of BMP-2 was 5'-GAAGCCAGG TGTCTCCAAGAG-3', and the reverse primer 5'-GTGGATGTCCTTTACCGTCGT-3'. Reactions were carried out in a reaction volume of 20 uL containing cDNA (2 uL), forward and reverse primer each (0.8 uL), SYBR Green PCR Master Mix (2X) (10 uL), and ddH_2O (7.2 uL). An Applied Biosystems 7500 quantitative PCR system was implanted by centrifugation for amplification reaction, performed under the following conditions: predenaturation at 95°C for 30 seconds, denaturation at 95°C for 5 seconds, renaturation at 60°C for 34 seconds, extension at 60°C for 1 minute, with 30 cycles. Using β-actin was used as an internal reference gene. Relative expression mRNA of ALP, Col-1 and BMP-2 in each

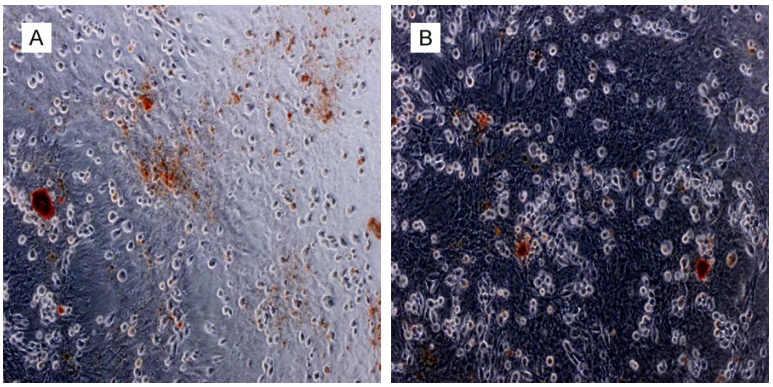

Figure 4. Alizarin red staining for osteogenic differentiation of BMSCs in the two groups cells (100×). A: Intermittent hypoxia group; B: Control group.

group were calculated by the $2^{-\Delta\Delta}$Ct method. Five samples were pre-specified for each group and averaged.

Statistical analysis

Statistical analyses of all experimental data were performed with the use of SPSS statistical software, version 21.0. Measurement data are represented as mean ± standard deviation and comparisons between the two groups were made by independent samples t-tests. Count data are expressed as percentages and the Chi-squared test was employed for comparisons between groups. A *P* value less than 0.05 indicates statistical significance.

Results

Culture and identification of rat BMSCs

One day after inoculation of the primary cells, some cells began to adhere to the surface of each well of the tissue culture plate. There were many mixed cells, mostly in the shape of a spindle. After 8 days of culturing, many cells adhered to the surface of each well, without evident mixed cells. Subculture was carried out until the third-generation cells were obtained. Purified cells were visualized, among which flat spindle cells were in great numbers (**Figure 2**). Cellular immunofluorescence staining showed that CD44, CD90 and CD105 were positively expressed, whereas CD34 and CD45 were negatively expressed (**Figure 3**).

Comparison of the formation of mineralized nodules in the two groups

Results of alizarin red staining showed a small amount of orange mineralized nodules were formed in the control group after 3 weeks of culturing. Many orange mineralized nodules were formed in the intermittent hypoxic group. The orange mineralized nodules were significantly more in the intermittent hypoxic group than in the control group (P<0.001; **Table 1** and **Figure 4**).

mRNA levels of osteogenic differentiation markers in the two groups of cells

Moreover, mRNA levels of ALP, Col-1 and BMP-2 in the intermittent hypoxia group were significantly higher than those in the control group at 24 hours, 48 hours and 72 hours, respectively (all P<0.01; **Figure 5**).

Discussion

OSAHS has been taken seriously by clinicians and researchers as a risk factor for various systemic diseases. OSAHS is primarily characterized as recurrent apnea accompanied by intermittent hypoxemia. Multiple studies have suggested that OSAHS affects, to some extent, osseous development and metabolism [16, 17]. Exploration of the mechanisms for the effects of OSAHS on osseous metabolism and bone density from the perspective of molecular biology is one of the current hotspots. The establishment of an OSAHS model is hard work in animal trials of OSAHS. Currently, it is relatively more stable to simulate the OSAHS state through the intermittent hypoxia mode in an external environment. To the best of our knowledge, few experimental studies have focused on the effects of intermittent hypoxia on changes in bone mineral density and microstructure, with relevant mechanisms of actions remaining unknown. BMSCs have potent proliferation and potential multidirectional differentiation. BMSCs have been shown to be differentiated into osteoblasts, *in vitro* and *in vivo*. Osteoblasts not only are a direct source of bone formation, but also regulate osteoclasts [18, 19]. The numbers and features of BMSCs are key in maintaining the normal physiological function of bones, at both skeletal development and repair stages [20]. Notably, exploring the effects of intermittent hypoxia on osteogenic differentiation of BMSCs allows a bet-

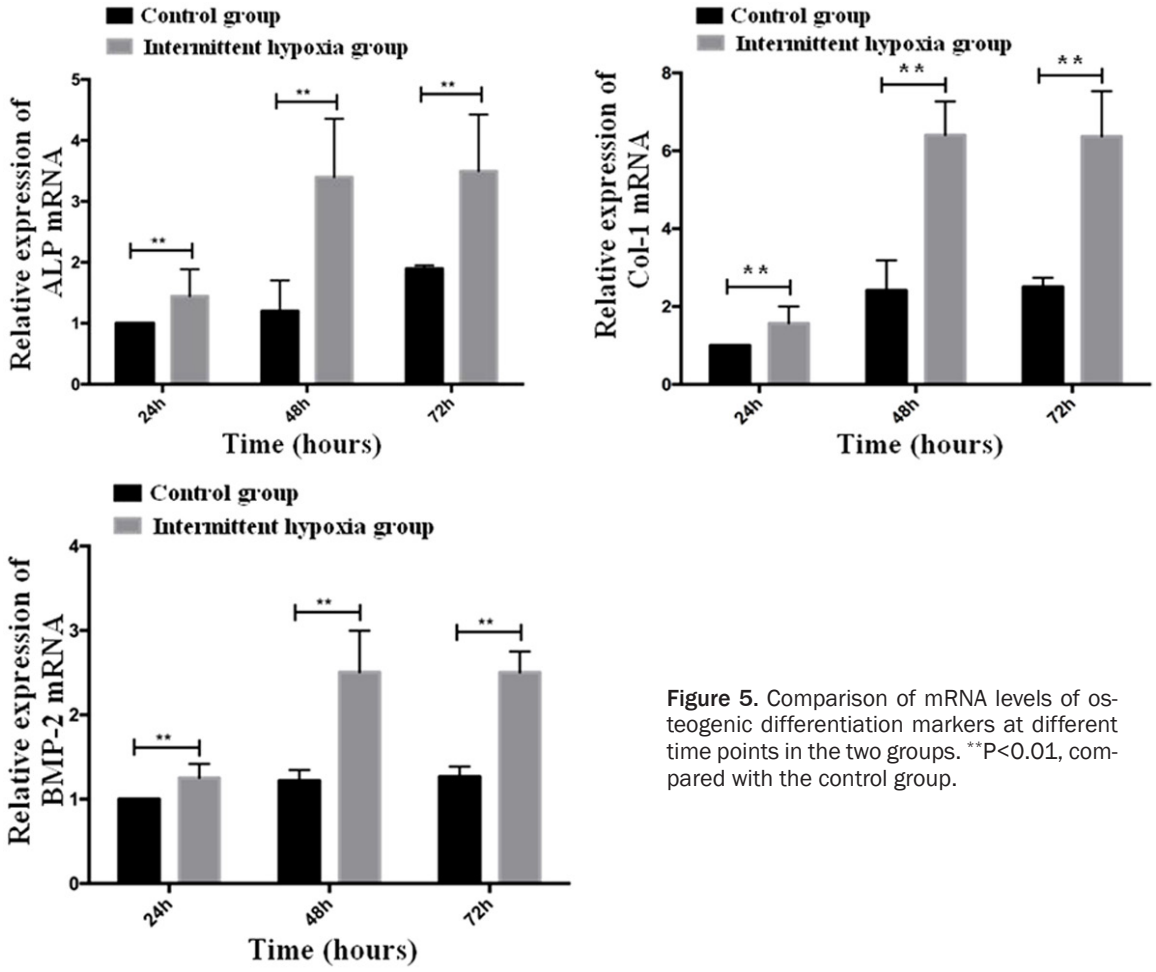

Figure 5. Comparison of mRNA levels of osteogenic differentiation markers at different time points in the two groups. **P<0.01, compared with the control group.

ter understanding of how OSAHS affects bone structure and morphological mechanisms.

One study argued that BMSCs are of low content in bone, accounting for only 0.001 to 0.01% of total bone marrow mononuclear cells [21]. Therefore, identification of BMSCs is very important. In the current study, two measures, adherent growth and expression of specific cell surface antigens (CD44, CD90, CD105, CD34 and CD45), were used for identification of BMSCs. Findings of our current study confirmed that acquisition and culture methods of BMSCs met the requirements of the experiment. One study reported that hypoxia promotes osteogenic differentiation of BMSCs, but another study argued that hypoxia inhibits osteogenic differentiation of BMSCs. Thus, the findings of studies vary greatly [22, 23]. The formation of mineralized nodules, which consist of collagen-affinity calcium salts secreted by osteoblasts, is one of the main markers for osteoblast differentiation and maturation. One study exploring the expression of calcium matrix and osteocalcin in primary rat BMSCs showed that levels of calcium matrix and osteocalcin in primary rat BMSCs induced in 5% hypoxia were significantly higher than those of primary BMSCs induced in normoxia (20%) [24]. Results of the current study showed that intermittent hypoxia increased the number of mineralized nodules in BMSCs, compared with the control group, suggesting that intermittent hypoxia induced differentiation of BMSCs into osteoblasts, consistent with that reported by Tsai et al. [25]. To delve into the effects of intermittent hypoxia on osteogenic differentiation markers in BMSCs, ALP, Col-1 and BMP-2 (osteogenic differentiation markers) were tested. ALP plays a crucial role in calcification *in vitro* and is an early marker for osteoblast differentiation. Is activity is also associated with the degree of osteogenic differentiation of BMSCs [26]. Col-1 is a major collagen com-

ponent in the bone matrix. Osteoblasts synthesize and secrete Col-1 and are involved in the formation of bone matrix [27, 28]. BMP-2, an osteogenic differentiation-promoting factor, induces differentiation of BMSCs into osteoblasts and facilitates repair of bone injury, a key cytokine that induces osteogenic differentiation of stem cells [29, 30]. The current study revealed that levels of ALP, Col-1 and BMP-2 (osteogenic differentiation markers) were significantly higher in BMSCs treated with intermittent hypoxia than those in the control group at 24 hours, 48 hours and 72 hours, respectively (all $P<0.01$). Results are in accord with those reported by Dong et al. [31].

In summary, intermittent hypoxia increased the number of mineralized nodules in BMSCs, upregulated levels of ALP, Col-1 and BMP-2 (osteogenic differentiation markers), and promoted the differentiation of BMSCs into osteoblasts. However, the mechanisms for differentiation of BMSCs are complicated. There may be multiple signaling pathways or target sites. Hypoxia induces HIF-1α expression and downregulates ROS levels and oxidative stress to affect osteogenic differentiation of BMSCs. Additional studies are necessary to investigate the specific mechanisms of action for intermittent hypoxia in the above processes, aiming to provide more experimental evidence for guidance of clinical treatment.

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Disclosure of conflict of interest

None.

Address correspondences to: Xiaofeng Lu, Department of Oral and Craniomaxillofacial Surgery, Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai City, P.R. China; Shanghai Key Laboratory of Stomatology and Shanghai Research Institute of Stomatology, Shanghai City, P.R. China; National Clinical Research Center of Stomatology, No. 639 Zhizaoju Road, Shanghai City 200011, P.R. China. Tel: +86-021-53315142; Fax: +86-021-53315142; E-mail: lukexf@163.com

References

[1] Lal C, Siddiqi N, Kumbhare S and Strange C. Impact of medications on cognitive function in obstructive sleep apnea syndrome. Sleep Breath 2015; 19: 939-945.

[2] Pham LV and Schwartz AR. The pathogenesis of obstructive sleep apnea. J Thorac Dis 2015; 7: 1358-1372.

[3] Sozer V, Kutnu M, Atahan E, Caliskaner Ozturk B, Hysi E, Cabuk C, Musellim B, Simsek G and Uzun H. Changes in inflammatory mediators as a result of intermittent hypoxia in obstructive sleep apnea syndrome. Clin Respir J 2018; 12: 1615-1622.

[4] Ma L, Zhang J and Liu Y. Roles and mechanisms of obstructive sleep apnea-hypopnea syndrome and chronic intermittent hypoxia in atherosclerosis: evidence and prospective. Oxid Med Cell Longev 2016; 2016: 8215082.

[5] Upala S, Sanguankeo A and Congrete S. Association between obstructive sleep apnea and osteoporosis: a systematic review and meta-analysis. Int J Endocrinol Metab 2016; 14: e36317.

[6] Song L, Liang X and Zhou Y. Estrogen-mimicking isoflavone genistein prevents bone loss in a rat model of obstructive sleep apnea-hypopnea syndrome. Int J Clin Exp Pathol 2014; 7: 1687-1694.

[7] Terzi R and Yilmaz Z. Bone mineral density and changes in bone metabolism in patients with obstructive sleep apnea syndrome. J Bone Miner Metab 2016; 34: 475-481.

[8] Tomiyama H, Okazaki R, Inoue D, Ochiai H, Shiina K, Takata Y, Hashimoto H and Yamashina A. Link between obstructive sleep apnea and increased bone resorption in men. Osteoporos Int 2008; 19: 1185-1192.

[9] Chen DD, Huang JF, Lin QC, Chen GP and Zhao JM. Relationship between serum adiponectin and bone mineral density in male patients with obstructive sleep apnea syndrome. Sleep Breath 2017; 21: 557-564.

[10] Carreras A, Almendros I, Acerbi I, Montserrat JM, Navajas D and Farre R. Obstructive apneas induce early release of mesenchymal stem cells into circulating blood. Sleep 2009; 32: 117-119.

[11] Carreras A, Rojas M, Tsapikouni T, Montserrat JM, Navajas D and Farre R. Obstructive apneas induce early activation of mesenchymal stem cells and enhancement of endothelial wound healing. Respir Res 2010; 11: 91.

[12] Carreras A, Almendros I, Montserrat JM, Navajas D and Farre R. Mesenchymal stem cells reduce inflammation in a rat model of obstructive sleep apnea. Respir Physiol Neurobiol 2010; 172: 210-212.

[13] Bianco P, Riminucci M, Gronthos S and Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 2001; 19: 180-192.
[14] Gozal E, Sachleben LR Jr, Rane MJ, Vega C and Gozal D. Mild sustained and intermittent hypoxia induce apoptosis in PC-12 cells via different mechanisms. Am J Physiol Cell Physiol 2005; 288: C535-542.
[15] Donaldson JG. Immunofluorescence staining. Curr Protoc Cell Biol 2015; 69: 4.3.1-7.
[16] Swanson CM, Shea SA, Stone KL, Cauley JA, Rosen CJ, Redline S, Karsenty G and Orwoll ES. Obstructive sleep apnea and metabolic bone disease: insights into the relationship between bone and sleep. J Bone Miner Res 2015; 30: 199-211.
[17] Schiza SE, Bouloukaki I and Mermigkis C. Bone mineral density in patients with obstructive sleep apnea syndrome. Sleep Breath 2013; 17: 17-18.
[18] Zhang P, Dai Q, Ouyang N, Yang X, Wang J, Zhou S, He N, Fang B and Jiang L. Mechanical strain promotes osteogenesis of BMSCs from ovariectomized rats via the ERK1/2 but not p38 or JNK-MAPK signaling pathways. Curr Mol Med 2015; 15: 780-789.
[19] Chen X, Wang J, Yu L, Zhou J, Zheng D and Zhang B. Effect of concentrated growth factor (CGF) on the promotion of osteogenesis in bone marrow stromal cells (BMSC) in vivo. Sci Rep 2018; 8: 5876.
[20] Gao Y, Li C, Wang H and Fan G. Acceleration of bone-defect repair by using A-W MGC loaded with BMP2 and triple point-mutant HIF1alpha-expressing BMSCs. J Orthop Surg Res 2015; 10: 83.
[21] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.
[22] Grayson WL, Zhao F, Izadpanah R, Bunnell B and Ma T. Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. J Cell Physiol 2006; 207: 331-339.
[23] Potier E, Ferreira E, Andriamanalijaona R, Pujol JP, Oudina K, Logeart-Avramoglou D and Petite H. Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. Bone 2007; 40: 1078-1087.
[24] Lennon DP, Edmison JM and Caplan AI. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. J Cell Physiol 2001; 187: 345-355.
[25] Tsai CC, Chen YJ, Yew TL, Chen LL, Wang JY, Chiu CH and Hung SC. Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through down-regulation of E2A-p21 by HIF-TWIST. Blood 2011; 117: 459-469.
[26] Mukaiyama K, Kamimura M, Uchiyama S, Ikegami S, Nakamura Y and Kato H. Elevation of serum alkaline phosphatase (ALP) level in postmenopausal women is caused by high bone turnover. Aging Clin Exp Res 2015; 27: 413-418.
[27] Yang X, Zhang H, Wang J, Zhang Z and Li C. Puerarin decreases bone loss and collagen destruction in rats with ligature-induced periodontitis. J Periodontal Res 2015; 50: 748-757.
[28] Wei QS, Huang L, Tan X, Chen ZQ, Chen SM and Deng WM. Serum osteopontin levels in relation to bone mineral density and bone turnover markers in postmenopausal women. Scand J Clin Lab Invest 2016; 76: 33-39.
[29] Grgurevic L, Macek B, Mercep M, Jelic M, Smoljanovic T, Erjavec I, Dumic-Cule I, Prgomet S, Durdevic D, Vnuk D, Lipar M, Stejskal M, Kufner V, Brkljacic J, Maticic D and Vukicevic S. Bone morphogenetic protein (BMP)1-3 enhances bone repair. Biochem Biophys Res Commun 2011; 408: 25-31.
[30] Wyatt LE, Chung CY, Carlsen B, Iida-Klein A, Rudkin GH, Ishida K, Yamaguchi DT and Miller TA. Bone morphogenetic protein-2 (BMP-2) and transforming growth factor-beta1 (TGF-beta1) alter connexin 43 phosphorylation in MC3T3-E1 cells. BMC Cell Biol 2001; 2: 14.
[31] Dong M, Jiao G, Liu H, Wu W, Li S, Wang Q, Xu D, Li X, Liu H and Chen Y. Biological silicon stimulates collagen type 1 and osteocalcin synthesis in human osteoblast-Like cells through the BMP-2/Smad/RUNX2 signaling pathway. Biol Trace Elem Res 2016; 173: 306-315.