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Midazolam suppresses osteogenic differentiation of human bone marrow-derived mesenchymal stem cells

T. ZHANG, H. SHAO[1], K.-Q. XU, L.-T. KUANG, R.-F. CHEN, H.-H. XIU

Department of Anesthesiology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China
[1]Department of Anesthesiology, the First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Tao Zhang and *Hua Shao* contributed equally to this work

Abstract. – OBJECTIVE: Previous study showed that peripheral-type benzodiazepine receptors (PBRs) are expressed in human mesenchymal stem cells (hMSCs) and diazepam was found to inhibit hMCSs viability in high concentration. Midazolam, a benzodiazepine derivative, is widely used as an intravenous sedative in hospital. Peripheral-type benzodiazepine receptors (PBRs) affect a broad spectrum of cellular functions. We tested the cell viability and osteogenic differentiation of hMSCs.

PATIENTS AND METHODS: Bone marrow was collected from 12 patients during the operation of spine internal fixation. Cultivated with basal medium, the hBMSCs were incubated with or without midazolam (0.1, 1, 5, 10, 15, 20 μM, respectively). Cell viability were tested with MTS assay after 2, 4, 6 hours respectively. Cell morphology was observed and recorded at 6 hour. After cultivated with osteogentic medium, the hBMSCs were incubated with or without midazolam (5, 10, 15, 20 μM, respectively). Alkaline phosphatase (ALP) activity and alizarin red S staining were measured. Cultivated with osteogentic medium with or without treatment of 15 μM midazolam, the mRNA expression of ALP, type 1 collagen (COL1), Runx2 and PPARγ was analyzed by real-time RT-PCR.

RESULTS: The treatments of midazolam inhibited cell viability to 85%-16% respectively ($p < 0.05$). Rounded up phenomenon with floating cells, Membrane-blebbed cells and cytoplasmic contraction were observed after 10, 15 or 20 μM midazolam treatment. The ALP activity and Calcium deposition of hBMSCs exposed to 15 and 20 μM midazolam was significantly inhibited at 7, 14 and 21 days ($p < 0.05$). And the mRNA expression of ALP, COL1 and PPARγ was significantly suppressed in the hBMSCs cultured with 15 μM midazolam ($p < 0.05$).

CONCLUSIONS: Midazolam exert negative effect on cell viability and osteogenic differentiation of cultured hBMSCs. During sedation in critical care, the use of midazolam may suppress activity of hBMSCs.

Key Words:
Midazolam, Apoptosis, Osteogenic differentiation, hBMSCs.

Introduction

Peripheral-type benzodiazepine receptors (PBRs) were found to be expressed in human mesenchymal stem cells (hMSCs), and diazepam, which is a mixed-type agonist of benzodiazepine receptors, had a depressing effect on hMCSs viability in high concentration[1]. However, whether midazolam could affect the osteogenic differentiation process of hBMSCs is unknown.

Midazolam, a benzodiazepine derivative, is widely used as an intravenous sedative in hospital due to its Pharmacokinetics and pharmacodynamics[2]. Previous studies suggested that midazolam activate both central and peripheral benzodiazepine receptors. Practically sedative effects of the benzodiazepines result from their actions on the central benzodiazepine receptors (CBRs) coupling with ionotropic $GABA_A$ receptors in the central nervous system[3]. In contrast to CBRs, peripheral-type benzodiazepine receptors (PBRs) which were discovered in 1977 affect a broad spectrum of cellular functions, such as regulation of steroidogenesis, modulation of cell growth and differentiation and apoptotic process[3]. It was revealed that midazolam can suppress some kinds of cell function such as TNF-α-induced endothelial activation and maturation of murine dendritic cells[4-6].

Mesenchymal stem cells (MSCs) are defined as undifferentiated multipotent precursor cells capable of self-renewal and differentiation[7]. The osteogenic differentiation of MSCs has been exten-

Corresponding Author: Kang-Qing Xu, MD; e-mail: tougaosci@aliyun.com

sively studied. Differentiation from MSCs into osteoblasts is known to be regulated by a number of molecular factors and mechanical stimulations. When cultured under the appropriate conditions, human mesenchymal stem cells (hMSCs) express several key osteogentic genes in a sequence progressing from osteoprogenitors to preosteoblasts, osteoblasts and osteocytes, suggesting that this process is critical for bone formation or remodeling.[8] In specific bone diseases such as trauma, congenital anomalies, osteoporosis and tumor, bone formation or remodeling is incomplete without a sufficient number of bone progenitors.

Aim

The present study was designed to investigate the effects of different concentration of midazolam on cell viability and osteogenic differentiation of hBMSCs.

Patients and Methods

Isolation and Cultivation of MSCs

Informed consent was obtained from the patients for bone marrow collection, and all the procedures were performed with the approval of the Research Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University, China. Bone marrow was collected from the drilling holes of pedicle during the operation of spine internal fixation of 12 patients (age range 41-60 years; mean age 48 years, 7 males and 5 females, including degenerative lumbar spondylolisthesis, disc herniation with lumbar spondylolisthesis, and lumbar spinal stenosis with instability). The patients were not receiving treatments that might affect bone marrow cells such as glucocorticoids or sex steroids. And the patients did not receive midazolam before and during the anesthesia. Primary cultures of mesenchymal stem cells were established by a modification of previously described methods[9]. Mononuclear cells were collected by Ficoll density gradient centrifugation (1.077; GE Health, Pittsburgh, PA, USA) from the bone marrow. The isolated cells were resuspended in basal medium and cultivated in flasks coated with fibronectin at 37°C with 5% CO_2 in humidified air. Basal medium consisted of Dulbecco's modified Eagle's medium with Glutamix-1, sodium pyruvate, 1000 mg/l glucose and pyridoxine (DMEM, Gibco, BRL, Grand Island, NY, USA) supplemented with 10% inactivated fetal bovine serum (FBS, Australian origin; Bio Whittaker Europe, Verviers, Belgium; Lot 8SB0001). After 72 hours, the medium was changed in order to remove any non-adherent cells. Thereafter, fresh culture medium was added and half of the medium was replaced every two days until subconfluence.

Measurement of Cell Viability (MTS Assay)

Cells were cultured at a density of 4×10^3 cells/cm^2 in flat bottomed 96-well plates (Techno Plastic ProductsAG, Trasadingen, Switzerland). One duplicate plate were supplemented with saline + medium and served as a positive control. In each other plate, duplicate wells were incubated with midazolam (Ehwa Pharma Corporation, China) at concentrations of 0.1, 1, 5, 10, 15 or 20 μM randomly. After 2, 4, 6 hours respectively, CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA) was added to each well according to the manufacturer's instructions. Two hours later, the culturing cell viability was determined by measuring the absorbance at 490 nm using a 550 BioRad platereader (Bio-Rad, Hertfordshire, UK).

Morphological Observation

The hBMSCs (4×10^3 cells/cm^2) were seeded in 96-well plates with 10% serum basal medium for overnight. Cells were treated without or with 0.1, 1, 5, 10, 15 and 20 μM midazolam randomly for 6 hours. Cell morphology was then observed and recorded using Olympus CK40 light microscopy (Olympus, Hamburg, Germany) at 100× magnification. Cell death was characterized by the loss of cellular contact with the matrix and the appearance of plasma membrane blebbing.

Osteogenic Differentiation

The hBMSCs were washed three times with phosphate buffered saline (PBS) and digested with 0.125% trypsin/5 mM EDTA digestion solution. After the cells were counted microscopically, they were detached and placed in 96-well plates at a density of 4000 cells per well in basal medium. After 24 hours, without or with various concentrations of midazolam (5, 10, 15, 20 μM), the culture medium was mixed with 0.05 mM ascorbic acid, 10 nM dexamethasone, 10 mM β-glycerophosphate (OS medium).

Alkaline Phosphatase Activity Assay

At day of 7, 14 and 21, cells in plates without or with midazolam (5, 10, 15, 20 μM, respective-

ly) were washed with PBS. 1.5 M 2-amino-2-methyl-1-propanol (Sigma-Aldrich, St. Louis, MO, USA, cat. no. A9226) was added as a buffer and incubated for 10 min. The substrate p-nitrophenyl phosphate (Sigma-Aldrich, cat. no. P5994) was added and incubated for 15 min. Finally 2M NaOH was added and incubated for 10 min to stop enzymatic reaction. The absorbance of he nitrophenolate produced was measured by microplate reader at 405 nm. ALP value was quantified by comparison with a standard curve made from the known concentrations of the substrate.

Values were normalized to optical density values determined by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt (WST-8) assay kit (CCK-8, Dojindo, Japan) according to the manufacturer's instructions and results are thus expressed in nmol/min/OD450. Briefly, WST-8 was added into each well for 1 hours before the ALP buffer was added. The absorbance at 450 nm was measured using a microplate reader.

Alizarin Red S Staining and Calcium Concentration Determination

At day of 14 or 21 after cultured with osteogenic medium, cell culture medium was removed. Then, cells were washed with PBS, fixed with ice-cold 70% ethanol for 1 h, rinsed in ddH_2O, and stained with 40 mM Alizarin Red S (ARS, Sigma-Aldrich, cat. no. A5533) at pH 4.2. To minimize nonspecifically bound stain, the cells were then rinsed with ddH_2O 5 times. The red staining of alizarin red S was identified under light microscope and photographed. Quantitative calcium deposition was measured by adding 10% cetylpyridinium chloride (CPC, Sigma-Aldrich, cat. no. c5460), then absorbance of solution at 560 nm was measured. ARS concentrations were calculated by comparison with an ARS standard curve and expressed as mg/ml.

RNA Extraction and Quantitative Polymerase Chain Reaction

After cultured with osteogenic medium, the cells were then washed three times with PBS and digested with 0.125% trypsin/5 mM EDTA digestion solution. After the cells were counted microscopically, they were detached and placed in 96-well plates at a density of 4000 cells per well in basal medium. After 24 hours, the culture medium was mixed with 0.05 mM ascorbic acid, 10 nM dexamethasone, 10 mM β-glycerophosphate (OS medium) treated without or with 15 μM midazolam for 7, 14 and 21 days. Total RNA in the hBMSCs cultured in OS medium was isolated using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was prepared from 1 μg of total RNA with cDNA synthesis kit (RevertAid™ H Minus First Strand cDNA Synthesis Kit, Fermentas, Hanover, MD, USA) in a final volume of 20 μl. Quantitative PCR was performed using a LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland) and the SYBR Green qPCR Master Mix (2X) (Fermentas, USA). Synthesized cDNA was diluted to 100 folds. The primers for all assayed genes were purchased from Invitrogen. Nucleotide sequences of PCR primers: GAPDH (Glyceraldehyde-3-phosphate dehydrogenase): Sense: 5`- GCACCGTCAAGGCTGAGAAC-3`; Antisense:5`-TGGTGAAGACGCCAGTGGA-3`(131basepairs) ALP: Sense:5`-GCCATTGGCACCTGCCTTAC-3`; Antisense:5`-AGCTCCAGGGCATATTTCAGTGTC-3`(137basepairs). COL1: Sense:5`-CTGCTGGACGTCCTGGTGAA-3`; Antisense:5`-ACGCTGTCCAGCAATACCTTGAG-3`(131basepairs). Runx2: Sense:5`-CACTGGCGCTGCAACAAGA-3`; Antisense:5`-CATTCCGGAGCTCAGCAGAATAA-3`(127basepairs). PPARγ: Sense:5`-GCCTGCATTTCTGCATTCTG-3`; Antisense:5`-CACGGAGCTGATCCCAAAG-3`(175basepairs). 11 μl of diluted cDNA was used in a single PCR reaction amounting to 25 μl. Analysis of the results was carried out using the software supplied with the machine. The software calculates each gene expression relative to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeper gene (delta CT) and then relative to controls (delta delta CT) using the fluorescence threshold of the amplification reaction and the comparative CT method. Each of the cDNA was tested in duplicate.

Statistical Analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Each data point in the figures represents the mean±SD of six separate experiments. Statistically significant differences between treatments and controls were determined by one-way ANOVA and then Least Significance Difference (LSD) comparison procedure. $p < 0.05$ was considered statistically significant.

Results

Effect of Midazolam on Cell Viability of hBMSCs

Figure 1 demonstrated the decreasing trend of cell viability in hBMSC treated with midazolam. In comparison to controls, midazolam revealed a significant decreasing effect on cell viability in a dose-dependent manner ($p < 0.05$). In comparison to controls, The treatment of 5 μM midazolam caused a significant decreasing effect on cell viability after 4h and 6h treatments (remaining 85% and 81% cell viability) ($p < 0.05$). Higher dose of midazolam profoundly reduced cell viability even in short time treatment. The treatments of 10, 15 and 20 μM midazolam decreased cell viability to 76%, 69% and 21% after 2h treatment ($p < 0.01$).

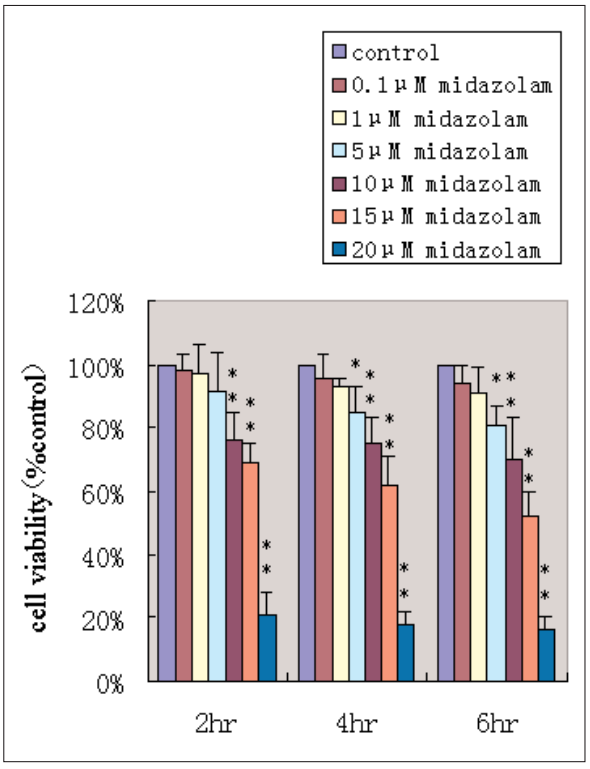

Figure 1. Inhibitory effects of midazolam on cell viability of hBMSCs. Midazolam was added to cultured hBMSCs cells and incubated for 2, 4 and 6 hours, respectively. Cell viability was assayed by MTS assay. The optical density of each well was measured at 490 nm using a microplate reader. The integrated absorbance value of control was regarded as 100%. Each data point in the figure represents the mean ± SD in percentage of cell viability from six independent experiments. Significant differences between the group treated with midazolam and the control group at the same time: $*p < 0.05$, $**p < 0.01$.

Effect of Midazolam on Morphological Changes of hBMSCs

Human bone marrow mesenchymal stem cells with lower dosage (0.1, 1 and 5 μ M) of midazolam (Figure 2b-d) for 6h treatment showed a spindle-shaped, fibroblast-like morphology with firm attachment, which is analogous to normal cell growth phenomenon (Figure 2a, control group). Rounded up phenomenon with floating cells were observed after 10 or 15 μM midazolam treatment (Figure 2e,f). Membrane-blebbed cells and cytoplasmic contraction were observed at the concentration of midazolam 20 μM (Figure 2g).

Effect of Midazolam on ALP Activity

As shown in Figure 3, the cells treated with 20 μM midazolam displayed lower ALP activity than the hBMSCs cultured in OS medium supplemented with saline (positive control) for 7, 14 and 21 days ($p < 0.01$). The ALP activity of hBMSCs exposed to 15 μM midazolam was significantly inhibited by 14 and 21 days ($p < 0.05$ or $p < 0.01$). However, the hBMSCs cultured in the lower dose of midazolam (5 and 10 μM) did not significantly reduce ALP activity compared with control group.

Effect of Midazolam on ARS Staining and Calcium Deposit

To further examine the effect of midazolam on osteoblast maturation, we performed ARS staining assay. A decreasing trend of calcium deposition was detected by quantitative analysis of ARS in the midazolam group compared to the control group. 15 and 20 μM Midazolam inhibited mineralization at 14 and 21 days (Figure 4).

Effect of Midazolam on Gene Expression During Osteogenic Differentiation

The expression of the gene markers in the hBMSCs is shown in Figure 5. The mRNA expression of ALP, COL1 and PPARγ was significantly suppressed in the hBMSCs cultured for 7, 14 and 21 days in the osteogenic media with 15 μM midazolam compared with the control medium (Figure 5a, b and d, $p < 0.05$ or $p < 0.01$). Expression of Runx2 in hBMSCs did not differ among the group treated with midazolam compared to the control group (Figure 5c, $p > 0.05$).

Discussion

In the present study, the effect of midazolam, a widely used benzodiazepine in clinical anes-

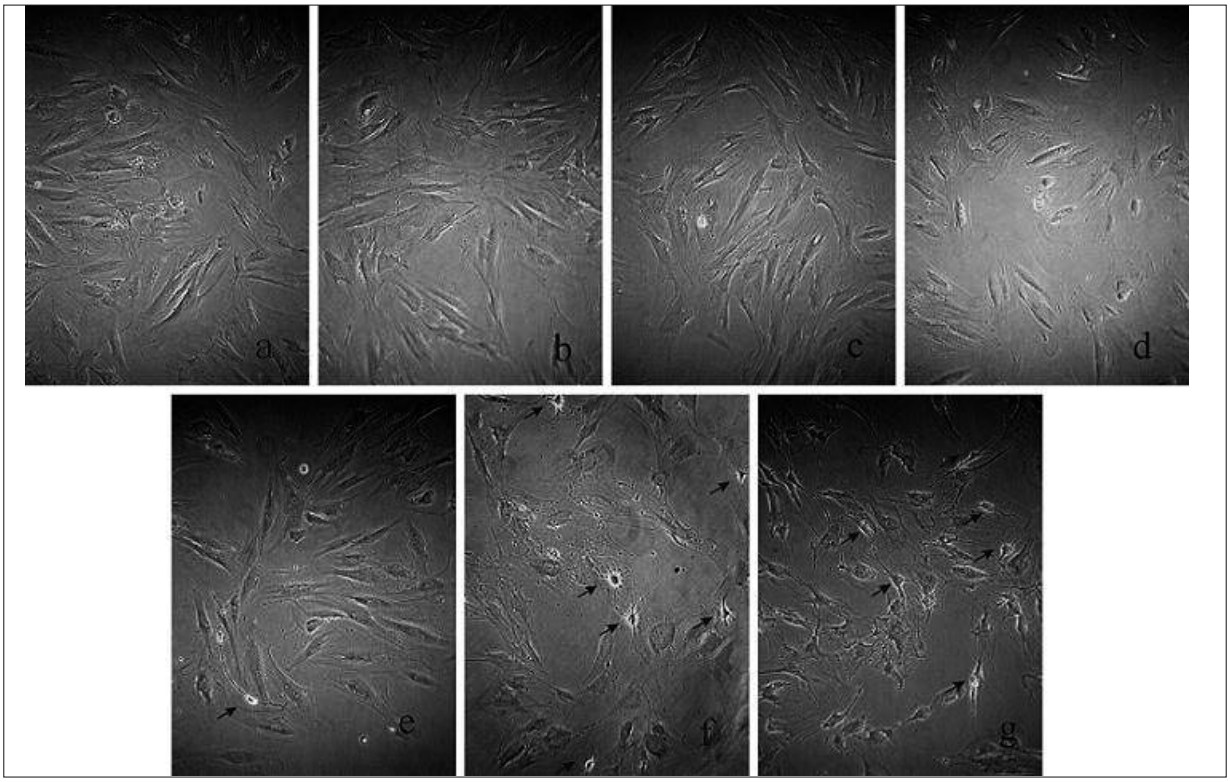

Figure 2. The effect of midazolam-induced morphological changes in hBMSCs. Cells were cultured in 96-well plates overnight and then treated without ***(a)*** or with 0.1, 1, 5, 10, 15, 20 μM midazolam ***(b-g),*** for 6 hours. Morphological changes of cells were examined under light microscopy. Arrow (↑) indicates membrane-blebbed cells.

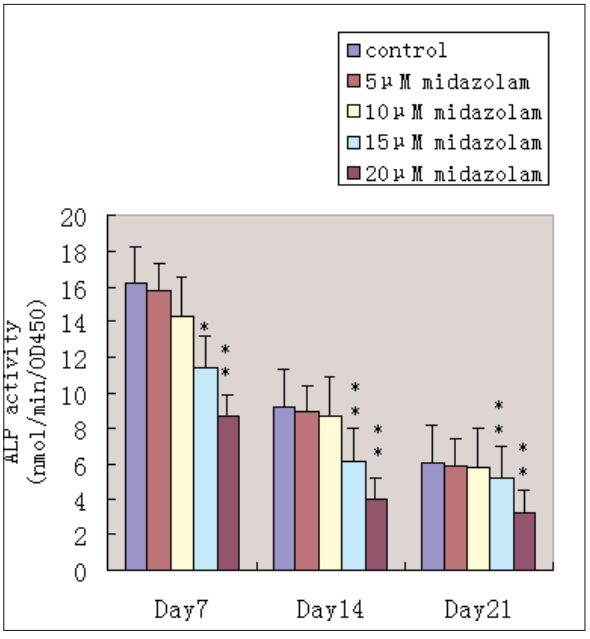

Figure 3. ALP activity in hBMSCs under osteogenic conditions treated with various doses of midazolam for 7, 14 and 21 days were analyzed. The ALP activity in hBMSCs of midazolam group was compared to the control group in six independent experiments (mean ± SD, *$p < 0.05$, **$p < .0.01$).

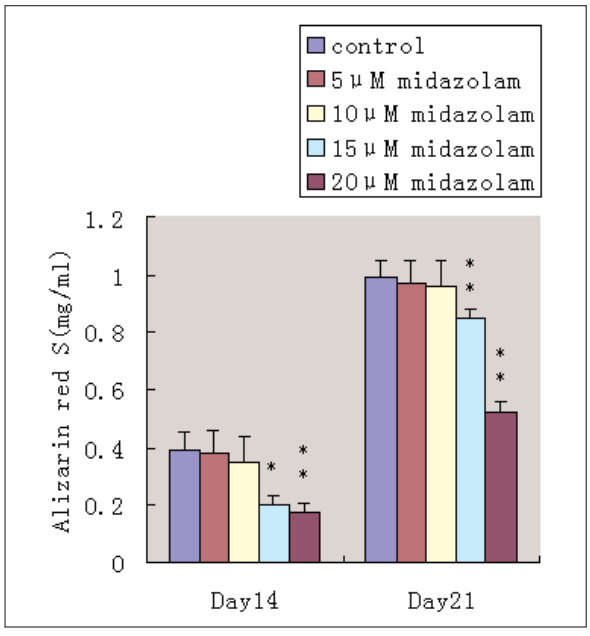

Figure 4. hBMSCs treated with midazolam exhibited less mineralization during osteogenic differentiation. Quantification of Alizarin red S with midzolam (5, 10, 15 and 20 μM) under osteogenic condition showed significantly decreased compared with control group at 14 or 21 days in six independent experiments (mean ± SD, **$p < .0.01$).

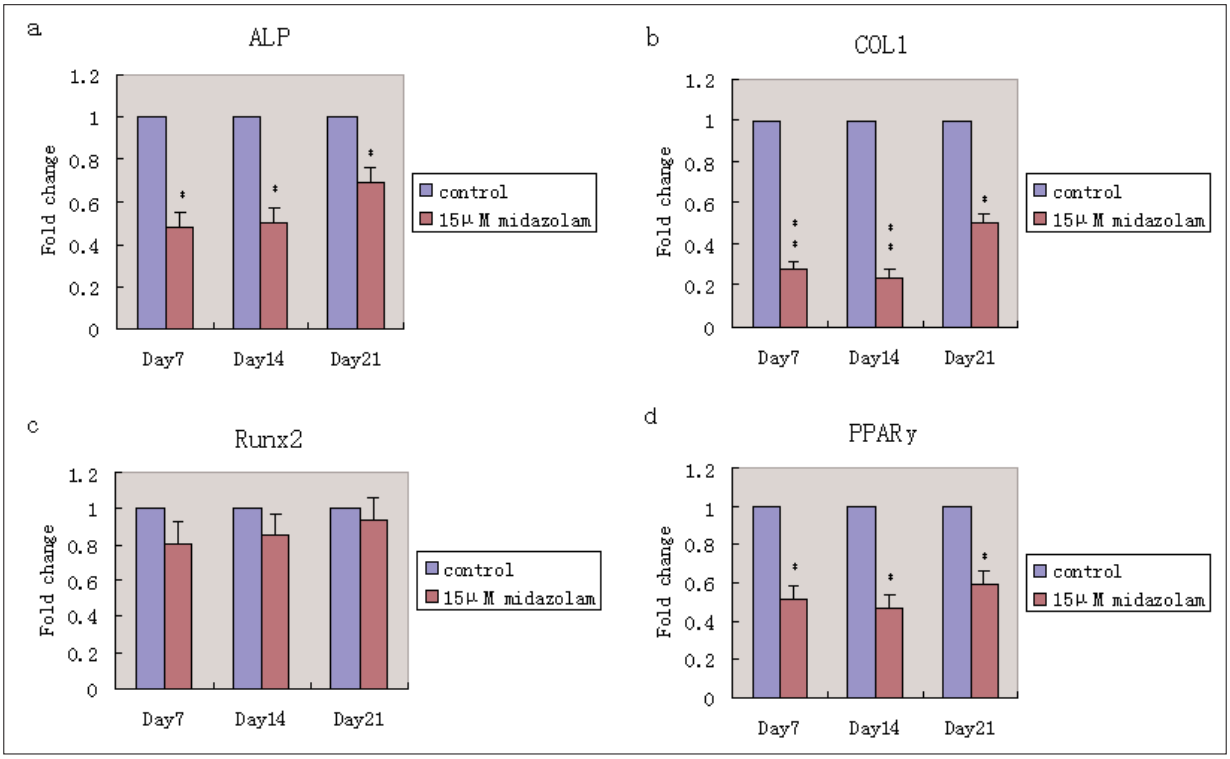

Figure 5. Quantitative analysis of osteoblastic markers gene expression when hBMSCs were cultivated in osteogenic medium without or with 15 μM midazolam for 7, 14 and 21 days. Q-PCR reactions were performed using primers for osteoblastic genes, including ALP ***(a),*** COL1 ***(b)***, Runx2 ***(c)***, and PPARγ ***(d)***. Data are expressed as the fold ratio relative to the expression of the respective gene in the hBMSCs cultured in control medium after normalization to GAPDH (*$p < 0.05$, **$p < 0.01$).

thesia, on hBMSCs was investigated in regard to cell viability and osteogenic differentiation *in vitro*.

The data of this study showed that midazolam reduced cells viability of hBMSCs in dose dependent manner. The cell viability of hBMSCs cultured with up to 5 μM midazolam for 4 and 6 hours was decreased significantly. Higher dose (10, 15 and 20 μM) of midazolam profoundly reduced cell viability after 2h treatment. Our study also showed that midazolam applied in high concentrations (10, 15 and 20 μM) would induce cell membrane blebbing or cytoplasmic contraction, which may lead to apoptotic cell death[10]. These results conform to a previously published report which showed that diazepam, which is also a mixed-type agonist of benzodiazepine receptors, had a depressing effect on hMCSs viability in high concentration[1].

ALP activity was used as a marker for osteogenic differentiation. In our investigation, cells cultured in the presence of the osteogenic differentiation medium with or with out midazolam had a continued decrease of ALP activity throughout the 21 days. Nevertheless, hBMSCs cultured supplemented with midazolam in the range of 15-20 μM had a lower ALP activity than cultured in control during the entire trial period. In addition, according to our results, a decreasing trend of mineral deposition was detected by quantitative analysis of Alizarin Red S in the midazolam (15, 20 μM) group compared to control group. As we know, terminal differentiation of human mesenchymal stem cells induced by the addition of osteogenic supplements can result in calcium deposition of the extracellular matrix as shown in Alizarin red S staining. In a pharmacokinetic study of midazolam-based sedation, EC_{50} concentration for sedation was reported to be 6.75 μM (2,200 ng/ml)[11]. But in clinical situations where deep sedation is necessary (e.g., therapeutic hypothermia), midazolam concentrations can exceeding 30 μM (10,000 ng/ml)[12]. Thus, it is possible for clinically used midazolam to inhibit osteogenic differentiation.

According to our result that ALP activity and mineral deposition were suppressed after treatment of up to 15 μM midazolam, we chose to test

the effect of 15 μM midazolam on the mRNA expression of hBMSCs *in vitro*. We found that midazolam (15 μM) directly inhibited the mRNA expression of ALP and COL1, which conformed to its inhibitory effect of ALP activity and mineral deposition in our study[13]. However, midazolam (15 μM) did not suppress the mRNA expression of Runx2 in hBMSCs. It is generally considered that Runx2 (CBFA1, AML3) are the major transcription factor controlling osteoblast commitment and differentiation[14]. Runx2 can regulate many but not all genes expression of osteoblasts such as type I collagen (COL1), osteocalcin (OC), and bone sialo protein (BSP) resulting in the establishment of an osteoblast phenotype[10]. One interpretation of our failing to find midazolam reduced stem cell gene expression of Runx2 is that the inhibitory effect of midazolam may via other transcriptional factors, such as osterix which acts downstream of Runx2 during bone development and regulates gene expression of COL1[15]. Moreover, midazolam might inhibit osteogentic differentiation by blocking the pathway of later stages of mesenchymal stem cell differentiation rather than inhibiting osteogenesis through early effects on progenitor cell. Alternatively, midazolam might preferentially influence downstream regulators such as signal transduction molecules other than transcription factors in regulation of commitment and differentiation.

Interestingly, we also found that midazolam decreased gene expression of peroxisome proliferator activated receptor γ (PPARγ) which is a critical factor for adipogenesis[16]. PPARγ is expressed in MSCs. It can bind Runx2 and inhibit its transcriptional activity[17]. This may explain our outcome of failing to find midazolam reducing stem cell gene expression of Runx2 on the other hand.

However, the findings of our cultured cell studies are of questionable relevance to *in vivo* conditions found in whole animals. Further, the activities of bone forming (osteoblast) may be only one of several factors contributing to the maintenance of stable bone mass. Therefore, further studies are required to testify the effect of midazolam on bone metabolism on the *in vivo* system.

Conclusions

Our data suggested that midazolam exert negative effect on cell viability and osteogenic differentiation of cultured hBMSCs. During sedation in critical care, the use of midazolam may suppress activity of hBMSCs.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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