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Midazolam enhances osteogenic differentiation of human bone marrow mesenchymal stem cells

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Abstract

Background: Bone remodeling involves a balance between osteoblast-driven formation and osteoclast-mediated resorption, with disruptions leading to diseases like osteoporosis. Midazolam (MDZ), known for its sedative properties, has shown effects on cellular differentiation and hydroxyapatite formation in dental cells. However, its role in promoting osteogenic differentiation in human bone marrow mesenchymal stem cells (hBMSCs) remains unexplored, motivating this study to investigate its potential in bone regenerative therapies.

Methods: Human bone marrow stem cells were cultured in the complete media with α-MEM, 10% FBS, and 1% pen/strep. Cell viability was determined with MTT assay in different concentrations of MDZ (0.125 to 1 µM) for 72 hours. Osteogenic differentiation was induced over 21 days using the selected doses of MDZ with osteogenic medium. The Alizarin Red S (ARS) staining was performed to determine the calcium deposit for osteoblast cells. Data were analyzed using repeated measure ANOVA, and a p-value <0.05 was considered statistically significant.

Results: The MTT results for different concentrations of MDZ (0.125 to 1 µM) showed no significant cytotoxic effects on hBMSCs after 72 hours. Furthermore, ARS staining revealed increased calcium deposits in 0.5 µM MDZ compared to untreated and DMSO groups (P-Value =0.0001). These findings suggest that MDZ promotes mineralization at lower concentrations, highlighting its potential in osteogenic applications.

Conclusion: Midazolam promotes osteogenic differentiation of hBMSCs, particularly at 0.5 µM concentration, without cytotoxic effects. These findings demonstrate that MDZ may be a potential compound for osteoblastogenesis; however, these findings require further in vivo studies to confirm the idea.

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Highlights

What is current knowledge?

Midazolam promotes osteogenic differentiation and calcium deposition.

What is new here?

Midazolam promotes differentiation of hBMSCs into osteoblasts.

Introduction

Osteoporosis is one of the most common bone disorders. It is characterized by accelerated bone resorption and decreased bone density, which results in increased susceptibility to fracture. As a silent progressive condition, osteoporosis represents a significant and growing medical challenge with extensive social and economic consequences worldwide. Addressing this public health concern requires early detection, effective prevention strategies, and targeted interventions to reduce its impact on individuals and healthcare systems (1,2). Moreover, bone formation occurs through the contribution of two important bone cells, including osteoblasts and osteoclasts, which are involved in bone formation and resorption, respectively, and tightly regulate processes responsible for continuous bone remodeling. Osteoclasts originate from hematopoietic stem cell precursors (HSCs) alongside the myeloid differentiation lineage (3), whereas osteoblasts are derived from a common progenitor cell with adipocytes and human bone marrow mesenchymal stem cells (hBMSCs) (4,5). The imbalance between bone formation and resorption may cause several bone diseases, such as osteopetrosis, osteopenia, and osteoporosis (3). The commitment of the mesenchymal stem cell (MSC) lineage to osteoblasts needs more detailed studies, not only because they share a common progenitor but also because of their important role in the BM microenvironment. A study by Lee et al. revealed that some benzodiazepine drugs, such as diazepam, inhibit adipogenesis at high concentrations and stimulate osteogenesis in the BMSCs at low concentrations. These findings support the effects of the benzodiazepine family of drugs and its derivatives on BMSC differentiation (6).

Midazolam (MDZ) represents a chemically synthesized derivative of imidazobenzodiazepine, characterized by its multifaceted pharmacological effects, including hypnotic, sedative, anesthetic, anxiolytic, muscle relaxant, and anticonvulsant effects (7).

Additionally, research focused on MSCs demonstrated that MDZ has a disadvantageous effect on cell viability and osteogenic differentiation in hBMSCs (8). Several in vitro investigations indicated the effect of MDZ on tumor, and cancer cells have revealed that MDZ promotes cellular apoptosis through the modulation of the caspase pathway, endoplasmic reticulum stress, autophagy, and cell cycle arrest (9-12). Furthermore, Karakida et al. suggested that MDZ enhances the differentiation of a porcine dental pulp-derived cell line (PPU-7 cell line) into odontoblasts and the formation of hydroxyapatite that closely resembles dentin in dental science (13). However, the proliferative effect of MDZ on the differentiation of hBMSCs remains poorly understood. In this study, we investigated the impact of MDZ on the osteogenic differentiation of hBMSCs.

Methods

Reagents

We purchased hBMSCs (Passage 3) from Royan Institute (Tehran, Iran). Fetal Bovine Serum (FBS), αMEM media, penicillin/streptomycin, Alizarin Red S (ARS) staining, and DMSO reagent were obtained from Idea Zist company (Iran, Tehran). Midazolam was provided from Daru-Pakhsh company (Iran, Tehran).

Cell culture and MTT assay

The hBMSCs were cultured in α-MEM containing 10% FBS and 1% penicillin/streptomycin for four days. Cells were detached using 0.05% trypsin for 10 min in a 5% CO2 incubator at 95% humidity (14,15). Cells were seeded at 1.0×10^6 for MTT assay in 96-well microplates. To determine the hBMSCs' viability, the cells were seeded at 1.0×10^4 density in each well of 96-well microplates. After four days and adaptation of cells, the complete α-MEM media was removed and the cells were divided into three groups, including the cells treated with different concentrations of MDZ (0, 0.125, 0.25, 0.5, and 1µM), cells were treated with 0.1% DMSO as the vehicle group, and untreated cells (Mock) for 72 hours. Finally, the MTT reagent (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well after 72 hours. The optical density (OD) was read after four hours using a plate reader at 450 nm (16,17) (Awareness microwell plate reader, USA).

Osteogenesis differentiation

After the identification of the optimal concentration of the MDZ, hBMSCs were treated for a period of 21 days using an osteogenic differentiation medium. The

complete differentiation medium included α-MEM as the basal medium, along with 100 nM dexamethasone, 200 µM ascorbic acid, 5 mM beta-glycerol phosphate, 1% Penicillin/Streptomycin, and 10% FBS (18). To determine mineralization and osteogenesis differentiation, hBMSCs were seeded in 12-well microplates at 5×10^4 cells/well. After 24 hours of adaptation, the cells were divided into three groups, including those cells treated with selected doses of MDZ, 0.1% DMSO (Drug vehicle) dissolved in osteogenic medium, as well as mock cells (Cells treated with basic medium) for three weeks (19). Furthermore, all treatments were carried out in triplicate. To determine osteogenesis and calcium mineralization in all groups, qualitative and quantitative ARS staining was carried out.

Alizarin Red S staining

After osteogenic induction for 21 days, based on the manufacturer's protocol, cell culture medium was removed from each well and washed gently three times with PBS (1X). Then, cells were fixed with 4% formaldehyde for 15 min at room temperature. The fixative was removed and washed three times with distilled water. Moreover, cells were stained with 1 mL of ARS stain (4 mM, pH 4.2) for 30 min. The ARS stain was removed and each well was washed five times with distilled water. Finally, extracellular mineralization was examined using a phase-contrast microscope. Furthermore, ARS quantification was determined as follows: The stained cells were washed with PBS and the mineralized nodules were dissolved in 10% acetic acid by gentle shaking. After neutralization with ammonium hydroxide, the supernatant was clarified by centrifugation. The absorbance of the supernatant was then measured using a microplate reader at 405 nm, and the ARS concentration was determined by comparison with a standard curve. The standard curve was constructed using serial dilutions of a 4 mM solution of ARS in hydrochloric acid (pH 4.0), from 2 to 0.0313 mM (19,20).

Statistical analysis

GraphPad Prism version 8.4 was used for all statistical analyses (GraphPad Software, United States). To determine the normality, we used the Shapiro-Wilk test. Statistical significance was analyzed through repeated measure ANOVA test when comparing more than two groups. In all cases, p-values of less than 0.05 were considered statistically significant. All experiments were performed in triplicate. For MTT assay, and quantitative ARS staining all values are presented as mean ± standard deviation (SD).

Results

Evaluation of cell viability in response to MDZ

After 72 hours of treatment of hBMSCs with MDZ, the results were compared to the mock and drug vehicle groups. The results revealed that treatment with MDZ had no toxic effect on the differentiation of hBMSCs into osteoblasts (P-Value = 0.504), compared to the control and the DMSO groups. Given that 0.5 and 1 µM concentrations were not toxic, they were selected for further investigations. The results are represented as mean ± SD. The obtained results showed p-values > 0.05 for all concentrations. The results indicated no toxic effects of MDZ doses on differentiation of BMSCs into osteocytes (Figure 1).

Assessment of calcium mineralization in osteogenic differentiation

The results of the Alizarin Red S (ARS) staining indicated that MDZ affected differentiation of hBMSCs into osteoblasts at concentrations of 0.5 µM and 1 µM, in comparison to the mock and DMSO control groups (Figure 2).

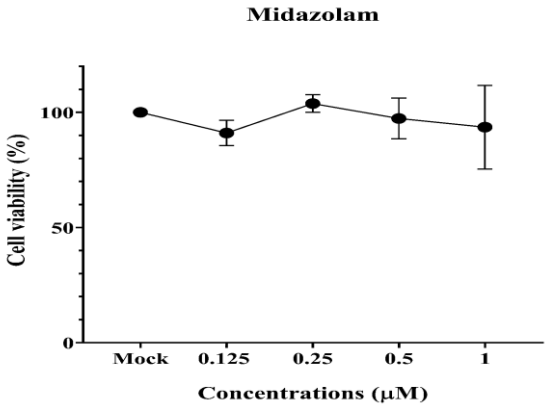

Figure 1. MTT assay results revealed cell viability under different midazolam (MDZ) concentrations (0.125, 0.25, 0.5, and 1 µM) over 72 hrs.

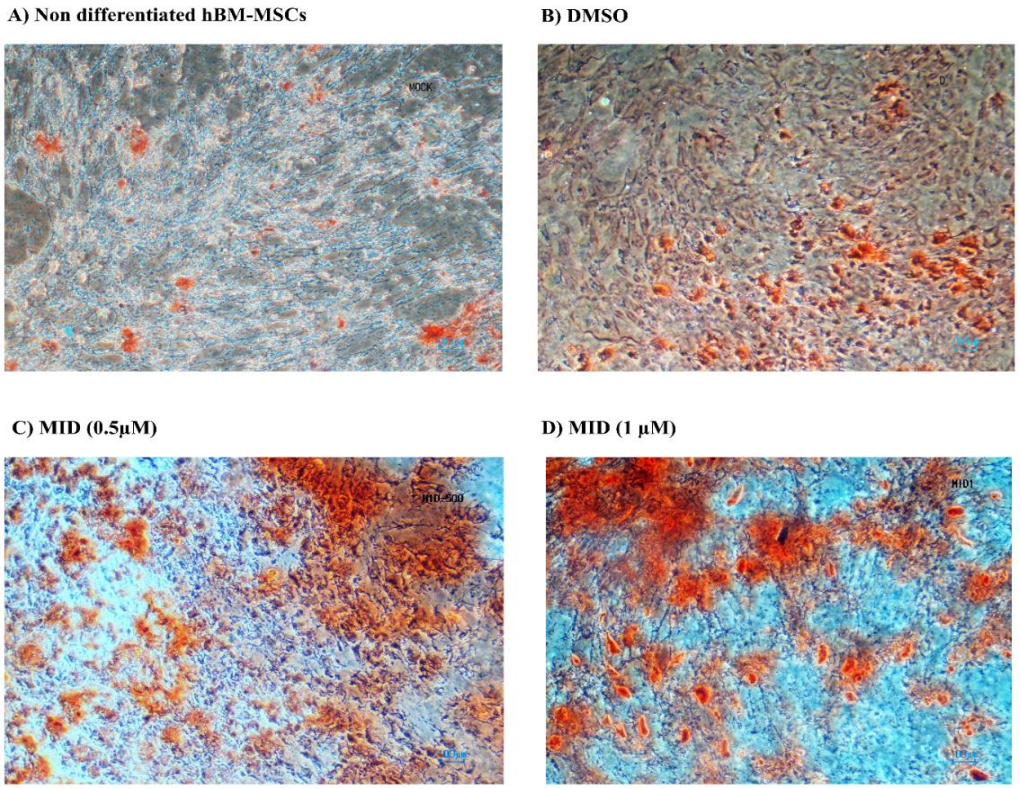

Figure 2. Qualitative ARS staining of human BMSCs without (A) osteogenic media (scale bar 100 µm); (B) hBMSCs with osteogenic media and DMSO (0.1%); (C), (D) BMSCs treated with osteogenic media and 0.5 and 1 µM concentration of MDZ. (C) and (D) shows the human osteoblast cells after 21-days of treatment in the presence of osteogenic media with MDZ concentrations (0.5 and 1 µM).

Quantitative measurement of Alizarin Red S staining in osteogenic differentiation

The ARS quantification staining of treated hBMSCs showed a significant difference between groups (P-Value = 0.0026). The results showed that the OD of ARS increased in DMSO (P-Value = 0.0056) and 0.5 µM MDZ (P-Value = 0.0034) groups compared to the undifferentiated cells. Furthermore, no significant difference was observed between 1 µM MDZ (p = 0.6594) and cells treated with DMSO. The results showed that 0.5 µM MDZ significantly increased extracellular calcium deposits, as evidenced by the characteristic orange-red staining of calcium deposits. This suggests that MDZ may act as a potent agent in enhancing the differentiation of hBMSCs into osteoblasts at low concentrations (Figure 3). The results demonstrated that 0.5 µM concentrations of MDZ markedly increased extracellular calcium mineralization, as evidenced by the intense orange-red staining characteristic of calcium deposits. This suggests that MDZ may serve as a potent agent in enhancing the differentiation of hBMSCs into osteoblasts in low concentrations, while higher concentrations had no differentiating effect on hBMSCs into osteoblasts. Results were demonstrated with mean ± SD. The results demonstrated a significant increase in osteogenesis following MDZ treatment (p =0.0034 for 0.5 µM concentrations of MDZ treatment and P-Value = 0.0056 for DMSO (0.1%)-treated cells compared to no treated groups).

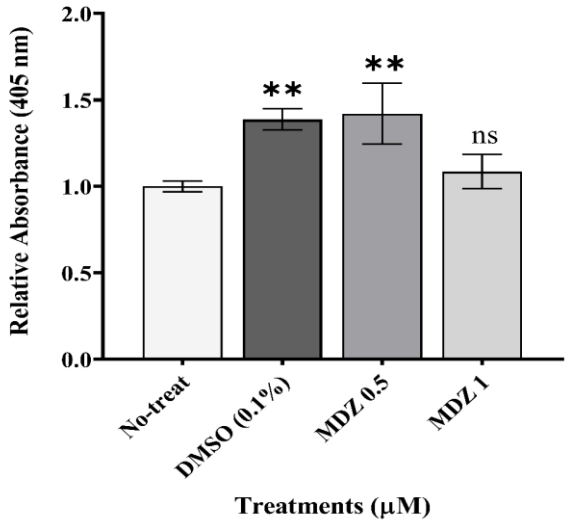

Figure 3. Quantification of ARS staining by absorbance at 405 nm. The groups were compared using repeated measure ANOVA test (treatments vs untreated and DMSO groups).

Discussion

Midazolam (MDZ), as a generic drug (21), in addition to its role in anesthesia and sedation (22), has recently been shown to induce osteogenic differentiation in dental pulp cells (13). However, there are also conflicting studies that have shown that MDZ can lead to a decrease in osteogenic differentiation in hBMSCs (8). Therefore, in the present study, the effect of different concentrations of MDZ on the differentiation of hBMSCs and formation of calcium nodules after 21 days of treatment was investigated.

The findings of the present study show that MDZ, at concentrations between 0.125 and 1 µM, exhibit no cytotoxic effects on hBMSCs after 72 h of treatment. These results align with the findings reported by Hidaka et al. (2020) who found no significant differences in C2C12 cell proliferation between MDZ-treated groups and controls (23). However, our results differ from those of Zhang et al. (2014) who reported a notable reduction in cell viability (16-85%) at higher concentrations (10, 15, and 20 µM) within 2-6 hours of treatment with MDZ (8). Harigaya et al. (2024) investigated the effects of different concentrations of MDZ (5, 10, 20, and 40 µM) on RAW264 cells over a six-day treatment. They revealed no evidence of cytotoxicity and increased osteoblastogenesis alongside a reduction in osteoclast differentiation (24), which aligns with the results obtained in our study. In light of these observations, the absence of cytotoxicity at lower concentrations of MDZ supports the potential therapeutic application of MDZ in hBMSCs.

Alizarin Red S staining, as a well-established method for assessing calcium deposition (Mineralization) (25), was used in hBMSCs treated with 0.5 and 1 µM MDZ concentrations for 21 days. Qualitative results of ARS staining in the treatment groups of hBMSCs treated with basal medium, 0.1% DMSO, 0.5 µM MDZ, and 1 µM MDZ in the presence of osteogenic differentiation medium showed that the OD of ARS sediment was higher in the treatment group with a concentration of 0.5 µM MDZ than in the treatment group with a concentration of 1 µM. In contrast, the OD was significantly lower in the sham and drug solvent groups. This finding indicates that MDZ at this concentration may effectively induce mineralization in hBMSCs, likely through promoting calcium-phosphate-based nodule formation. These results align with the study by Hidaka et al. (2020), which demonstrated that MDZ at a concentration of 500 ng/mL in combination with BMP-2 enhanced mineralization in C2C12 cells after ten days (23). However, our findings contrast with those of Zhang et al. (2014), who reported a decreasing trend in calcium deposition in MDZ-treated cells at higher concentrations (15 and 20 µM) compared to the control group after 14 and 21 days, as measured by quantitative ARS staining (8). These results may be attributed to differences in experimental conditions, such as the cell type, MDZ concentration, and culture duration. Furthermore, Karakida et al. (2019) revealed that treatment with 10 µM MDZ over a duration of ten days leads to mineralization in the PPU-7 cell line, which confirms our findings. While Karakida et al. treated UPP-7 cells with a higher concentration of MDZ (10 µM) for ten days, the present study took a different approach by administering a lower dose of MDZ (0.5 µM) for 21 days to facilitate osteogenic differentiation (13).

Our findings highlight the dose-dependent effects of MDZ on mineralization, suggesting that lower concentrations may optimize its pro-mineralization properties. However, further research is needed to focus on deciphering the underlying mechanisms of MDZ-induced mineralization and to investigate its combined effects with other osteogenic factors in hBMSCs. In the present study, we experienced several limitations such as accessibility to the other hBMSCs, osteogenic induction media, limited range of concentrations tested, and the lack of *in vivo* studies.

Conclusion

This study indicated that MDZ at low concentrations is non-cytotoxic and could effectively promote calcium mineralization in hBMSCs in 0.5 and 1 µM, highlighting its potential in regenerative medicine of bone tissue. However, future studies are necessary to focus on mechanisms of MDZ-induced mineralization and its potential in combination with osteogenic factors for clinical applications.

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Ethical statement

Ethical approval was obtained from the Research Committee of Golestan University of Medical Sciences with Ethics number IR.GOUMS.REC.1399.377.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Dr. J. Asadi provided supervision and guidance during the experimental work. Dr. M. Saghaeian Jazi supervised the experimental steps and participated in editing the manuscript. Dr. S. M. Jafari and Dr. S. M. Mir participated in editing the manuscript and responded to the referees' comments. Dr. M. Amanlou helped in study design. Dr. Z. Mohammadi participated in conducting the experiments, analyzing the data, and writing the manuscript.

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