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The Osteogenic Effect of Zingerone on Human Umbilical Cord Stem Cells via miR-590 and Smad7 Expressions

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ABSTRACT

Background and Objective: The Osteoblast differentiation is an essential process that causes bone stability and homeostasis. Zingerone (ZG) 4-(4-hydroxy-3-methoxyphenyl)-2-butanone isolated from the ginger plant is involved in many biological processes and can be used to treat diseases as an anti-inflammatory and antidiabetic agent. This study aims to identify ZG potential to bone tissue regeneration by investigating the effect of Zingerone on human umbilical cord stem cells (hUC-MSCs) and their differentiation into osteoblasts.

Methods: In this cross-sectional study, the effect of Zingerone toxicity on 2×10^6 hUC-MSCs cells was investigated in 4 groups; one control group without any ZG and 3 groups threated with 50µM, 100µM and 200µM ZG. Cell viability was evaluated by the MTT method after 24, 48 and 72 hours. The gene expression of miR-590 and Smad7 and differentiation markers such as Osterix (OSX) and runt-related transcription factor 2 (RUNX2) were investigated by quantitative real-time polymerase chain reaction (qRT-PCR). The expression level of this enzyme was checked by an alkaline phosphatase (ALP) reaction.

Findings: Zingerone has significant proliferative effects on hUC-MSCs cells in 200µM ($p<0.05$). Zingerone positively affects the differentiation process of osteoblasts by influencing the expression of specific markers such as ALP ($p<0.05$ in 200µM), RUNX2 ($p<0.001$ in 200µM), and OSX ($p<0.001$). The expression of miR-590 is increased in 200µM ($p<0.05$), while that of Smad7 is decreased under the influence of different Zingerone concentrations ($p<0.001$). In fact, miR-590 suppresses Smad7 and helps the differentiation of osteoblast cells.

Conclusion: The results of this study showed that Zingron causes the differentiation of osteoblast cells by increasing the expression of specific markers of osteoblasts (OSX, RUNX2 and ALP). In addition, increasing the expression of miR-590 suppresses Smad7 and helps the differentiation of osteoblast cells.

Keywords: *Zingerone, Osteoblast Differentiation, miR-590, Smad7, ALP*.

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Introduction

Mesenchymal stem cells (MSCs) are present in the bone marrow, umbilical cord, adipose tissue, and placenta in adults, and they maintain the reserves of these tissues (1). These cells can differentiate into different cells such as osteoblasts, cartilage, fat, nerve, and muscle cells. Umbilical cord Mesenchymal stem cells (UC-MSCs) have great practical value in medicine due to being separated from a broad and accessible source and the lack of ethical issues (2).

As a member of the body's skeletal and regenerative system, bones create the body's shape and support the body's mechanical, motor, and protective activities. In addition, they balance mineral ions and regulate metabolism. Maintaining the homeostasis of bones guarantees their various functions (3). The differentiation of mesenchymal stem cells into osteoblasts is the basis of maintaining bone homeostasis. Thus, discovering the differentiation pathways and the factors involved is of great significance (4).

Zingerone (ZG) 4-(4-hydroxy-3-methoxyphenyl)-2-butanone is obtained as a non-volatile compound as a result of drying ginger (5). There are several reports on the benefits of ZG in human health. It possesses anti-inflammatory, antioxidant and anti-genetic damage effects and also helps neutralize the anti-apoptotic effects induced by radiation (6). However, studies on the impact of ZG on osteoblast differentiation are quite limited. For example, in a study, the positive effect of ZG on the differentiation of mice mesenchymal stem cells into osteoblasts showed the unique role of this compound in restorative medicine (7).

MicroRNAs are single-stranded non-coding sequences of 18 to 22 nucleotides that bind competitively to the 3.UTR region of the target mRNA (8). Various studies show the role of different miRNAs in bone tissue maintenance and differentiation. For example, miR-96 is involved in the differentiation and formation of mice bone tissue through the Wnt signaling pathway (9). Moreover, miR-214 overexpression inhibits the differentiation of mesenchymal stem cells into osteoblasts by suppressing beta-catenin and weakening the Wnt/β-catenin pathway (10).

Recently, studies have shown that Smad7 inhibits TGF-β by affecting its receptor (11). The role of Smad7 has also been identified in tumorigenesis and metastasis of colorectal cancer, breast cancer, melanoma, and endometrial carcinoma (12). In addition, it can mediate TGF-β inflammatory responses and inflammatory autoimmunity. On the other hand, Smad7 participates in the differentiation of osteoblasts (13). However, the effect of ZG on osteoblast differentiation has not clarified yet. UC-MSCs are multipotent stromal cells that can differentiate into osteoblasts, chondrocytes, adipocytes, and other cells to threat different bone diseases upon stem cell-base therapy (14). In this study, we have identified the osteogenic effects of ZG on human umbilical cord MSCs at molecular levels and the expression of miR-590 and Smad7 in human umbilical cord stem cells.

Methods

This experimental study was designed and performed in Tehran Islamic Azad University and approved by the Research and Ethic Committee with reference number: IR.IAU.CTB.REC.1400.042. Human Umbilical Cord mesenchymal stem cells were purchased from Royan Research Institute's cell bank and incubated in a DMEM/F12 culture medium supplemented with 10% FBS at 37°C and an atmosphere of 5% CO2 and 95% air. Osteogenic cell induction was done with different concentrations of Zingerone. HUC-MSCs cells were incubated with 50, 100, and 200µM Zingerone concentrations for 72 hours. The group without Zingerone was considered as the control.

Investigating the effect of Zingron on the proliferation of stem cells derived from the umbilical cord by MTT method: MTT assay was performed to investigate the cytotoxicity of ZG. About 5×10^3 cells were cultured in 96-well plates and treated with the desired concentrations of Zingerone for 24, 48, and 72 hours to perform this test. Then the supernatant was removed, and 200µl of 0.05% MTT solution (Sigma-Aldrich) was added to each well. After incubation for 1 hour at room temperature, the supernatant was removed, and dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to each well to dissolve the formazan crystals. Finally, the optical density (OD) at 570 nm was measured by a spectrophotometer (Thermo Fisher).

Examining the expression of specific genes of osteoblasts by polymerase chain reaction method: Total RNA of hUC-MSCs treated with different concentrations of ZG for 72 hours was isolated using TRIzol reagent (Sigma-Aldrich). cDNA synthesis was done using a cDNA reverse transcription kit made by Pars Toos Company. The qRT-PCR reaction was performed using SYBR Green PCR Master Mix made in Amplicon Co. The relative expression of genes was calculated using the formula$2-\Delta\Delta$Ct. β-actin gene was used as the internal control. The primers of this study are listed in Table 1.

Table 1. Primers used in the differentiation of hUC-MSCs into Zingerone-treated osteoblasts

Genes	F and R Primers
RUNX2	CCCAGTATGAGAGTAGGTGTCC GGGTAAGACTGGTCATAGGACC
OSX	ACCCGTTGCCTGCACTCTC CACAATGTTCTCTCCCCAAGCT
miRNA-590	GGGGGAGCTTATTCATAAAA CAGTGCGTGTCGTGGAGT
Smad 7	TGTCCAGATGCTGTGCCTTCCT CTCGTCTTCTCCTCCCAGTATG
β-actin	GGCATCCTCACCCTGAAGTA TGAGTGTAAGGACCCATCGGA

Alkaline phosphatase level test (ALP test): After 14 days of hUC-MSCs treatment with different Zingerone concentrations, the cells were washed with PBS, lysed by NP4O lysing buffer, and centrifuged at 12000 rpm. The supernatant was separated, and alkaline phosphatase activity was tested at 450 nm using an alkaline phosphatase kit (Pars Azmoun, Iran) with the colorimetric method and spectrophotometer.

All experiments were repeated three times. The data obtained in SPSS software version 16 were analyzed as mean±SD using a one-way analysis of variance (ANOVA), followed by the Tukey-Kramer post hoc test. P-value of less than 0.05 was considered statistically significant.

Results

Zingerone has proliferative effect on hUC-MSCs cells: The results of MTT assay revealed that 50, 100, and 200µM concentrations of zingerone on hUC-MSC cells significantly have proliferative effects specially in 200µM compared to the control specimen ($p<0.05$) (Figure 1A). The morphology of the cells was spindle-shaped after treatment with Zingerone for 72 hours, and they were in favorable conditions for cell growth (Figure 1B).

Treatment of umbilical cord stem cells with Zingerone differentiates cells into osteoblasts: The expression of *RUNX2* and *Osterix* genes at the mRNA level was investigated by the qRT-PCR method to determine the osteogenic effect of ZG on hUC-MSCs cells. The results showed that after 72 hours of cell

treatment in different concentrations of ZG (50, 100, and 200µM), the expression of the *RUNX2* gene increased compared to the control, so the concentration of 200µM showed a significant difference compared to the control ($p<0.001$). In comparison to the controls, the *OSX* gene expression also increased with an increasing concentration of ZG ($p<0.001$) (Figure 2).

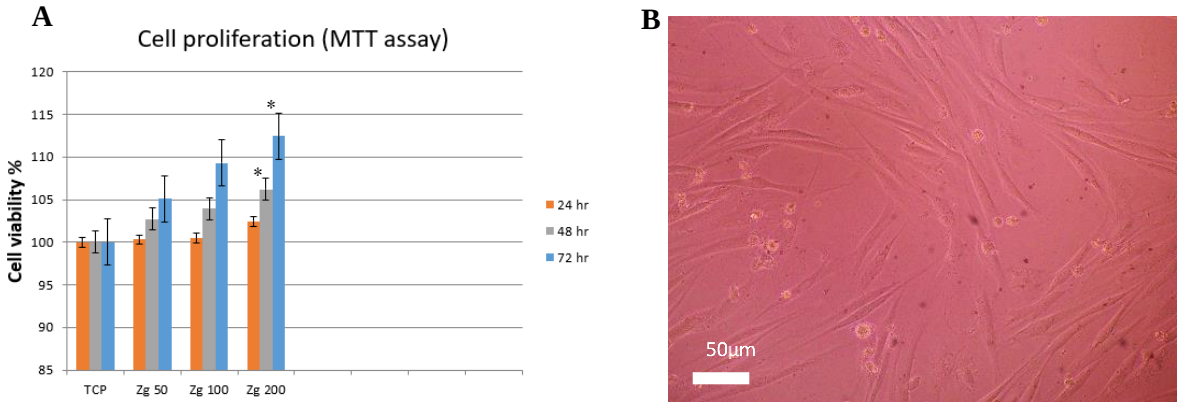

Figure 1. The effect of Zingerone on the survival and proliferation of hUC-MSCs cells. A: MTT assay results of the significant effect of proliferation of 200µM ZG on cells after 48, and 72h (*p<0.05). B: Cell morphology of hUC-MSCs after treatment with ZG after 72h.

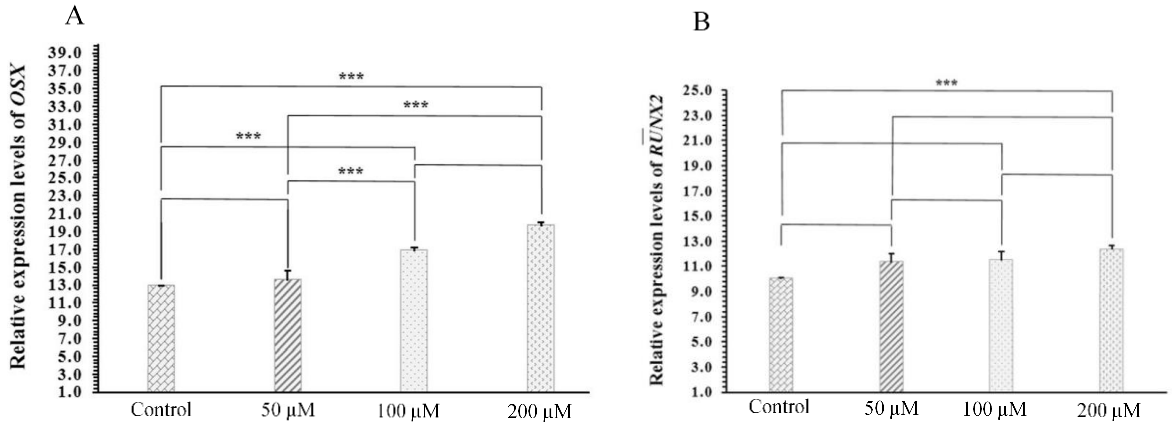

Figure 2. Osteogenic effect of ZG on hUC-MSCs cells at the mRNA level after 72 hours. A: The OSX gene expression in different concentrations of ZG (50, 100, and 200µM) increased significantly compared to the control group. B: The RUNX2 expression in different concentrations of ZG increased compared to the control. At the concentration of 200µM, a significant difference is observed compared to the control (*p<0.001).**

miR-590 expression increases while Smad7 expression decreases in UC-MSCs cells treated with different Zingerone concentrations: QRT-PCR test was performed to detect the effect of Zingerone on miR-590 expression. The results showed that miR-590 had increased expression in a Zingerone dose-dependent manner compared to the control. This increase in expression was especially significant at the concentration of 200µM compared to the control ($p<0.05$) (Figure 3A). The Smad7 expression decreased significantly in all doses compared to the control with increasing ZG concentration ($p<0.001$) (Figure 3B, C). These data showed that miR-590 and Smad7 were altered in Zingerone-induced UC-MSCs.

Expression of alkaline phosphatase in UC-MSCs cells treated with different concentrations of Zingerone: The osteogenic effect of Zingerone concentrations on hUC-MSCs cells after 14 days of culture showed that the expression of alkaline phosphatase enzyme increased with increasing Zingerone concentration, and this increase in expression was significant compared to the controls at a concentration of 200µM $p<0.05$ (Figure 4).

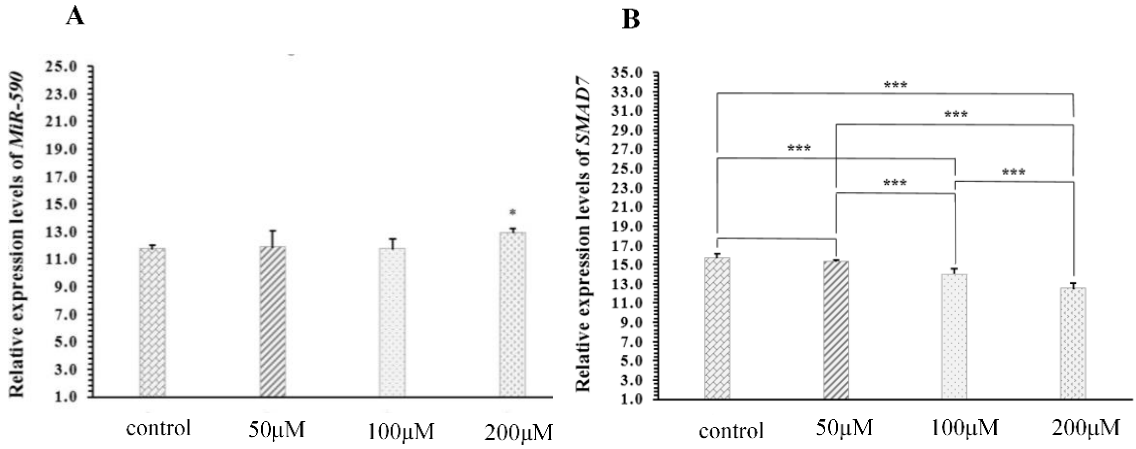

Figure 3. miR-590 expression increased, and Smad7 expression decreased with ZG. A: miR-590 expression in hUC-MSCs cells treated with different concentrations of ZG (50, 100, and 200µM). B: Smad7 expression in hUC-MSCs cells treated with different concentrations of ZG (50, 100, and 200µM). ($^{*}p<0.05$, $^{***}p<0.001$).

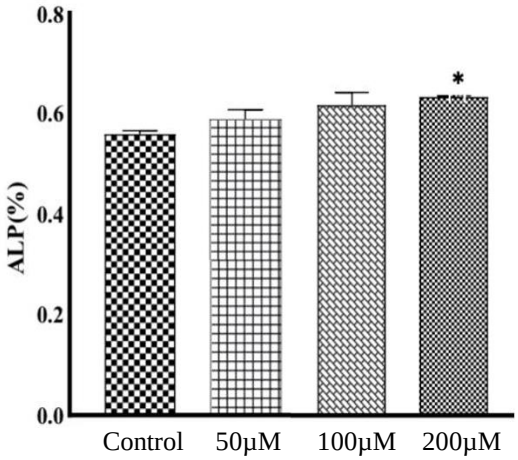

Figure 4. Increased expression of ALP under the influence of increased concentration of ZG in hUC-MSCs cells after 14 days. At the concentration of 200µM ZG, the ALP expression increase was significant ($^{*}p<0.05$).

Discussion

This study evaluated the impact of Zingerone on hUC-MSCs cells and the effect of Zingerone treatment on the expression of osteoblast differentiation markers. Moreover, an increase in miR-590 expression and a decrease in Smad7 expression of the ZG-induced cells was observed. Considering the role of these two markers in the differentiation process of osteoblasts, these data reveal the possible mechanisms by which

ZG differentiates the osteoblastic cells (14). Due to the change in lifestyle, people are more inclined to use natural compounds to treat diseases (15). ZG is a natural compound separated from ginger during the drying process (16). Zingerone has many biological functions, inclusing anti-inflammatory, antioxidant, and anti-diarrheal effects (17). ZG has positive effects on the differentiation of mice mesenchymal stem cells into osteoblasts at the cellular and molecular level (18).

This study also showed that ZG is involved in cell differentiation into osteoblasts by stimulating the expression of differentiation markers such as *RUNX2*, *OSX*, and ALP in hUC-MSCs cells. *RUNX2*, expressed in osteoblasts and chondrocytes, is reported as an inducer of these cells' differentiation (19). ALP is considered a key marker in the early stages of osteoblastic differentiation (20). *OSX* is a zinc finger-containing transcription factor essential for bone formation and osteoblast differentiation (21). In agreement with previous studies, this paper revealed the differentiation function of ZG in osteoblasts, and this function was enhanced through the expression of miR-590 (22).

miRNAs play an essential role in regulating osteoblast differentiation by targeting *RUNX2*. For example, in one study, miR-30c is upregulated during osteoblast differentiation (23). New studies show that miR-590-5p promotes the differentiation of osteoblasts and indirectly causes the maintenance and stability of RUNX2 by targeting SMAD7 (24). In this study, treatment of hUC-MSCs cells with ZG increased the expression of miR-590, which indicated its possible role in regulating Smad7 and protecting RUNX2 in osteoblastic differentiation. Smad7 has a negative role in regulating TGF-β1 signaling and bone formation (25). Overexpression of Smad7 in mice preosteoblast cells decreases bone formation significantly. The negative effect of Smad7 on osteoblast differentiation is believed to be due to Smurf2-mediated reduction of RUNX2.

The results showed the effect of ZG on hUC-MSCs differentiation into osteoblasts through miR-590 on Smad7 and the protection of RUNX2. In conclusion, this study showed that ZG stimulates the differentiation of hUC-MSCs into osteoblasts at the cellular and molecular levels. According to the obtained data, the differentiation pathway of these cells is related to the regulatory path of miR-590/Smad7. Therefore, further study of this topic could pave the way to find better means to repair bone.

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