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Original article

Nobiletin promotes osteogenic differentiation of human osteoblastic cell line (MG-63) through activating the BMP-2/RUNX-2 signaling pathway

Ying Pang [a,*], Lili Liu [a], Hong Mu [a], Vishnu Priya Veeraraghavan [b]

[a] Dental Clinic, Cangzhou Central Hospital, Cangzhou City, Hebei Province 061000, China
[b] Department of Biochemistry, Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, 600 077, India

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ABSTRACT

Nobiletin (NOB) is polymethoxy flavonoids, which plentifully there in *Citrus depressa* and they demonstrate numerous pharmacological effects. NOB has an anti-proliferative effect, attenuates ovalbumin-treated eosinophilic airway inflammation and Type II collagen treated arthritis. NOB noticeably inhibits bone resorption and renovates bone loss in mice model, but role of NOB in bone metabolism is unclear. Human bone is a important organ that sustains its homeostasis among bone resorpting osteoclasts and bone developing osteoblasts. The balances of among these two kind of cell outcomes are implicated in bone remodeling. The current study designed to explore possessions of NOB on differentiation and proliferation of MG-63 cells and contribution of morphogenetic protein signaling. Cell proliferation was analyzed by MTT, mineralization analysis by alizarin red staining and morphogenetic signaling protein by RT-PCR. No stimulus outcome of NOB on cell proliferation was found at days of 1, 3 and 7. Accumulation of calcium was augmented after that treatment of NOB. The mRNA expression of BMP-2, COL-I, ALP, OCN, RUNX2 and COL1A1 augmented markedly with NOB supplement. Hence, NOB can stimulate osteogenic differentiation of MG-63, almost certainly by promoting RUNX2 and BMP-2 signaling and this result might provide to its action on stimulation of osteoblast development, differentiation and augments of bone mass.

1. Introduction

Human bone is a important organ and sustains its homeostasis among bone resorpting osteoclasts and bone developing osteoblasts. The dynamic balances between these two kinds of cell outcomes are implicated in bone remodeling (Macias et al., 2020). Osteoporosis is a decrease in bone accumulation due to a disparity amongst bone formation and bone resorption, while bone homeostasis need sense of balance connections among osteoclasts and osteoblasts (Wildman et al., 2019). Existing drugs are employed to cure osteoporosis consist of calcitonin, estrogen, bisphosphonates and ipriflavone. These are every one-bone resorption inhibitors, which sustain bone accumulation by suppressing roles of osteoclasts (Buckley et al., 2017). Although, effect of these compounds in growing or getting better skeletal mass is comparatively small and positively no more than 2% per annum (Pavone et al., 2017). Thus, to have acceptable bone building mediators like teriparatide, it promote new bone development and modify differences of trabecular microarchitecture features of established osteoporosis (Zhang et al., 2020).

The World Health Organization (WHO) regards as osteoporosis to be a most important health difficulty as it has an effect on over 200 million people globally (Tarrant and Balogh, 2020). Age-associated bone loss, which involves about 25% of men and 50% of women over 50 years old, is the mainly imperative lifetime risk factor for having an osteoporosis-associated fracture (Sozen et al., 2017). Bone developments is consisting of a complex sequence actions during the mesenchymal stem cells are making different into osteoblasts. Mediators, which control bone development, operate by either growing cell proliferation of osteoblastic lineage or activating osteoblasts differentiation (Kim et al., 2020). In process of osteoblast differentiation, bone morphogenetic proteins (BMPs) are involving an important task in bone development through formation of bone specific matrix proteins (Halloran et al., 2020). BMP-2, an imperative intensification marker in BMP

* Corresponding author.
E-mail address: poiuytre125@sina.com (Y. Pang).

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subfamily and regulates differentiation osteoblast through promoting osteoblast-associated transcription factors, containing alkaline phosphatase (ALP), osteocalcin (OCN), COL-I and RUNX2 (Phimphilai et al., 2006; Liu et al., 2014; Ying et al., 2014).

Nobiletin (NOB) is polymethoxy flavonoids, which plentifully there in *Citrus depressa* and they demonstrate numerous pharmacological effects. NOB has anti-proliferative property on different kinds of cancer cells (Akao et al., 2008), attenuates ovalbumin-treated eosinophilic airway inflammation in asthmatic rats (Wu et al., 2006) and Type II collagen treated arthritis (Murakami et al., 2007). NOB also controls adipocytokine secretion and adipocyte differentiation (Miyata et al., 2011). NOB noticeably suppress bone resorption and renovates loss of bone in ovariectomized mice (Harada et al., 2011), but roles of NOB in bone metabolism is unclear. For that reason, the current study designed to explore possessions of NOB on differentiation and proliferation of MG-63 cells and contribution of morphogenetic protein signaling.

2. Materials and methods

2.1. Chemicals and cell culture

NOB was procured from Sigma-Aldrich Chemicals Pvt. Ltd, USA. Dimethyl sulfoxide (DMSO), phosphate buffered saline (PBS), MTT, DMEM, penicillin G, streptomycin and fetal bovine serum (FBS) were attained from Himedia Lab Ltd, USA. MG-63 cells were purchased and maintained in DMEM including penicillin streptomycin (1%) and FBS (10%) at 37C and CO_2 (5%) to confluence (80%). The MG-63 cells were next harvested, cleansed and cells were additional sub-cultured and employed in all *in-vitro* tests.

Table 1
Primer sequences for target genes.

Target gene	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
OCN	CACTCCTCGCCCTATTGGC	CCCTCCTGCTTGGACACAAAG
ALP	GGCTCTGCCGTTGTTTC	GGGTTGGGTTGAGGGACT
COL-1	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
BMP-2	GGTATCACGCCTTTTACTGCC	ACACCCACAACCCTCCACAA
RUNX2	TAAATACGCAGCAGACCG	CAGCACCCTCCATCACTT
COL1A1	CTCAGCCCTCTGTGCCT	AACCTTCGCTTCCATACTC

2.2. MTT assay

The viability of MG-63 cells was appraised through MTT methods. The cells were seeded in 96 well plate and kept at 37 ℃ (24 h). The medium was next addition with varied dosages of NOB (0, 10, 20 and 30 μg/ml) and kept for 1, 3 and 7 days. Afterward, 10 μl of MTT (5 mg/ml) were combined to all well. Followed by plate was kept at 37 ℃ for 4 h to support insoluble formazan crystal development. Following incubation, the supernatants were removed and 100 μl of DMSO was employed to dissolve insoluble formazan and was measured at a wavelength of 570 nm.

2.3. Evaluation of mineralization by alizarin red-S (ARS) staining

To assess mineralization accumulations for bone nodule development and cellular matrix was stained with AR dye that mixes with Ca^{2+} in matrix. In brief, MG-63 cells were cultivated in 24-well plates and added with 10 and 20 μg/ml of NOB for 1, 3 and 7 days. The cells were cleansed twice with PBS and preset with paraformaldehyde (4%) for 30 min. Then, cells were stained with 40 mmol/L of AR solution (pH 4.4) for 5–10 min at RT.

2.4. RT-PCR analysis

Osteoblasts cells were cultivated in 6 well plates at a level of 1×10^6 cells/well. The cells were added with NOB at levels of 10 and 20 μg/ml for 1, 3 and 7 days. Total RNA from cells of all well was separated with TRIzol reagent by the manufacturer's information (Thermofisher, USA). RNA aliquots were reverse transcribed to cDNAs by the Transcriptor First Strand cDNA Synthesis Kit. The thermocycling comprises 1 cycle for 3 min at 95 °C, 40 cycles of 95 °C for 20 s, 60 °C for 40 s, 72 °C for 20 s, and 1 cycle of 72 °C for 10 min. The RT-PCR study was employed by SYBR Green Master Mix and the specific primers for OCN, ALP, COL-I, BMP-2, RUNX2 and COL1A1 (Table 1). Xeno™ RNA control was used as positive control and GAPDH was used as an internal control.

2.5. Statistical investigation

Results were statistically analyzed by using SPSS statistical software version 17.0. Data are articulated as means ± SD. One-way ANOVA subsequently DMRT assay was employed to evaluate find-

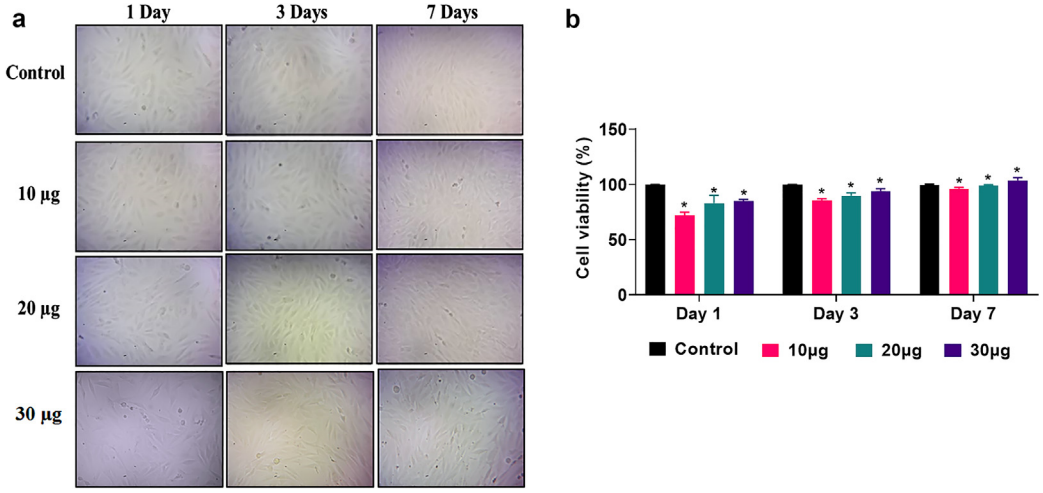

Fig. 1. Effect of NOB on the cell viability of MG-63 cells. The viability of MG-63 cells did not affected by the presence of NOB, which is investigated by optical microscopy on 1, 3 and 7 days. NOB efficiently promoted the viability of MG-63 cells. (B). NOB significantly increased the viability of human osteoblast-like MG-63 cells in dose dependent manner. MG-63 cells were treated with 10, 20, and 30 μg/mL of NOB for 1, 3, 7 days. Data were illustrated as mean ± SD. Note: *p < 0.05 when compared with the control.

ings among various groups. A P value < 0.05 was measured to point out a statistically significance of variation.

3. Result

3.1. Cytotoxicity effect of NOB on MG-63 cells

The impact of NOB on viability and growth of MG-63 cells was investigated with MTT investigation. Fig. 1A and B explained NOB was showed no major changes on cell viability in every tested dose (0, 10, 20 and 30 μg/ml), which demonstrating that NOB was no cytotoxic to MG-63 cells.

3.2. Effect of NOB on mRNA expressions of bone morphogenetic signaling proteins

The osteogenic effect of NOB on MG-63 cells was examined by RT-PCR. The expression of COL-I, ALP, RUNX2, COL1A1, BMP-2 and OCN mRNA expressions on MG-63 cells were shown in Fig. 2(A&B). The supplements with 10 and 20 μg/ml of NOB at 1, 3 and 7 days,

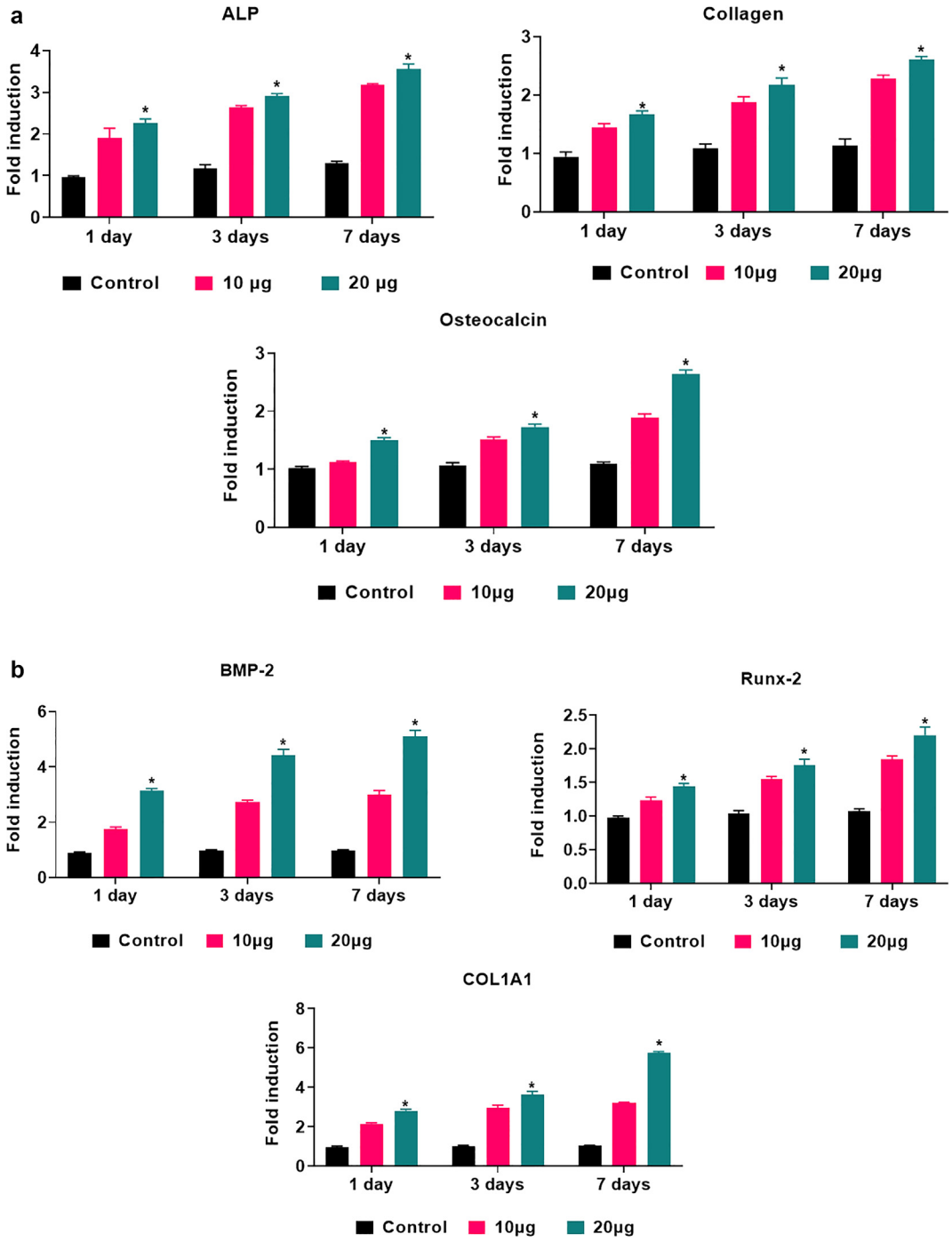

Fig. 2. Effect of NOB on the mRNA expression of ALP, Collagen, and Osteocalcin in the MG-63 cells. MG-63 cells were treated with 10 and 20 μg/ml of NOB and control cells for 1, 3 and 7 days are exposed. NOB up-regulated the expression of ALP, Collagen, and Osteocalcin in the MG-63 cells. Data were illustrated as mean ± SD. Note: *p < 0.05 when compared with the control. (B). MG-63 cells were treated with 10 and 20 μg/ml of NOB and control cells for 1, 3 and 7 days are exposed. NOB increased the expression of ALP, Collagen, and Osteocalcin in the MG-63 cells. Data were illustrated as mean ± SD. Note: *p < 0.05 when compared with the control.

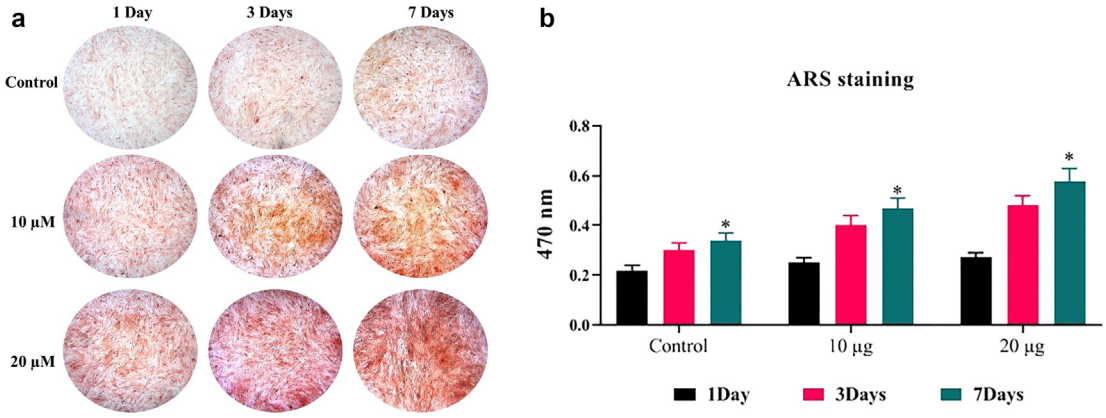

Fig. 3. Influence of NOB on mineralization in MG-63 cells. The calcium deposits in the mineralized matrix were investigated by alizarin red staining. NOB effectively increased the calcium deposition in the MG-63 cells. (B). MG-63 cells stimulated with the treatment of 10 and 20 μg/ml of NOB and control cells for 1, 3 and 7 days are exposed. NOB effectively increased the calcium deposition in the MG-63 cells. Data were illustrated as mean ± SD. Note: *p < 0.05 when compared with the control.

activated ALP gene expression, more than baseline levels considerably. Comparable to COL-I level mRNA expression was stimulated by NOB treatment at all concentrations at 1, 3 and 7 days. Moreover, OCN, RUNX2 and COL1A1 mRNA expression were markedly augmented by the administration of NOB as related to non-treated MG-63 cells in 1, 3 and 7 days.

3.3. Effects of NOB on mineralization in MG-63 cells

Mineralization is an essential for formation of bone. For this study, the significance of NOB for bone mineralization and quantitative assessment of mineralization was performed using ARS. The calcium accumulations were exhibited that supplements with 10 and 20 μg/ml of NOB considerably augmented the mineralization in MG-63 cells at 1, 3 and 7 days evaluated with the control cells (Fig. 3A and B). These data are pointed out that NOB can activate mineralization in MG-63 cells.

4. Discussion

Osteoblasts play an essential function in bone development and remodeling, though, these unsuccessful to formation of bone regrowth in musculoskeletal illness i.e., osteoporosis (Lin et al., 2020). In recent times, it has been recommended that plant-derived drugs exhibit a curative effect on osteoporosis (Barbalho et al., 2019). Harada et al. (2011) stated that NOB, a polymethoxy flavonoid are suppressing the bone resorption due to suppression of NFκB-associated prostaglandin E formation in osteoblasts and avert bone loss during estrogen insufficiency. Hence, in current work, the effects of NOB on osteoblastic MG-63 cells were evaluated. To explore results of NOB on bone metabolism, we provide work for a cell culture method employing MG-63 cells. MG-63 cells show evidence of osteoblast-like qualities like formation of OCN and COL-I (Bique et al., 2016). MG-63 cells provide as a helpful model for investigation of biological management of different materials (Ruiz-Gaspa et al., 2007). First, we analyzed whether NOB suppress proliferation of osteoblasts. NOB had no stimulus effects the proliferation of MG-63 cells. These results pointed out that NOB is harmless to MG-63 cells, which propose risk of decreasing side effects. Similarly, Yu et al. (2018) reported that Semaphorin 3A enhances osteogenesis through the upregulated proliferation of MG63 cells and did not showed any negative effects to MG-63 cells. Yoo et al. (2017) reported that Melanin extract from *Gallus gallus domesticus* promoted the cell proliferation and differentiation of MG-63 cells. Our findings from this study coincides with these repots.

BMPs are vital in controlling differentiation of osteoblast and consecutive bone development. BMP-2, has demonstrated powerful osteosynthesis effects *in vitro* and *in vivo* (Yao et al., 2020). In the current study, NOB was activating the BMP-2 pathway and osteogenic-associated genes. These findings recommended that BMP-2 signaling be implicated in NOB-treated cell differentiation, which is agreements with the earlier findings through Semaphorin 3A in MG-63 cells (Yu et al., 2018). RUNX2, an essential transcriptional mediator for differentiation of osteoblast, has been informed to be provoked by BMP-2. Additionally, RUNX2 directly interrelates with promoter regions of main osteoblast particular genes, containing ALP and OCN (Yao et al., 2020). In the current work, activities of NOB on RUNX2 expression in MG-63 cells was observed. The findings exhibited that RUNX2 was induced by NOB demonstrating that NOB provokes osteogenic differentiation. Previous study also reported that linarin activates osteogenic differentiation in MC3T3-E1 osteoblast cell line via the BMP-2/RUNX2 pathway (Li et al., 2016).

ALP expression is the majority commonly documented biochemical indicator for osteoblastic process and is considered to participate a task in bone mineralization (Kuo and Chen, 2017). COL1A1 is an early stage osteogenic differentiator marker, which directly stimulated by RUNX2 (Xie et al., 2015). For that reason, we observed the effect of NOB on ALP and COL1A1 levels of the MG-63 cells. NOB augmented ALP and COL1A1 mRNA expression due to the enhancing the proliferation and differentiation of osteoblasts, agrees with previous studies (Li et al., 2016). COL-I and OCN are most important extracellular protein substances of bone matrix which is critical for bone tissue development. OCN is expressed in late stage differentiation of osteogenic, therefore OCN and COL-I reveal the mechanism of osteoblastic growth (Gu et al., 2011; Huang et al., 2017). For that reason, we evaluated the biological efficiency of NOB on the COL-I and OCN actions of MG-63 cells. The findings of mRNA analysis were revealed that NOB added MG-63 cells expressed on higher levels of COL-I and OCN levels.

NOB provoked the development of osteoblasts and is supported for osteoblast mineralization. In concurrence with our findings, studied by Wang et al. (2019) proved *Artemisia annua* provokes osteoblast effects. Calcium deposition assessments were demonstrated the increased in the treatment of NOB in MG-63 cells, which agreements with the earlier report. Hyun et al. (2014) reported that Watercress containing Rutin augmented levels of mineralization with various concentrations in MG-63 cells. These

data reveal that the capability of NOB to increase matrix mineralization, which was positively related to the differentiation and proliferation of osteoblasts, demonstrating that NOB is valuable in suppressing osteoporosis by triggering osteoblast role (Trachootham et al., 2009; Zhu et al., 2017). In overall, our findings proved that the NOB provoked the development of osteoblasts and increased the mineralization. However, we appraised here our *in vitro* experimental findings only. The more in depth studies on animal models were still needed in the future to elucidate the therapeutic role NOB against bone related diseases.

5. Conclusion

In conclusion, NOB can enhance osteogenic activity by triggering osteogenic differentiation associated indicator gene synthesis in MG-63 cells. Though, it has no stimulatories outcome on MG-63 cell proliferation when NOB employed as a treatment in cell culture medium. NOB provoked untimely developments of osteoblasts and is supported for osteoblast mineralization. Moreover, osteoblastogenic effects of NOB was intervened by promoting BMP-2 reactive and RUNX2 signaling proteins.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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