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Original Article

EFFECT OF OSTERIX AND OSTEOCALCIN ENHANCEMENT BY QUERCETIN (3,3′,4′,5,7-PENTAHYDROXYFLAVONE) ON OSTEOBLAST HFOB 1.19 CELL LINE

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ABSTRACT

Objective: This study was aimed at investigating the effect of quercetin (3,3′,4′,5,7 -pentahydroxyflavone) as a phytoestrogen in the treatment of estrogen deficiency-induced osteoporosis, through the measurement of osterix and osteocalcin expressions on osteoblast hFOB 1.19 cell line.

Methods: hFOB 1.19 cells were cultured in 24-well microplates, induced with 10 ng/ml TNF-α and incubated for 24 h. TNF-α induction was used to create an estrogen deficiency condition. Quercetin was then added at 10 µM concentration. The immunocytochemistry double staining method was performed with anti-rabbit osterix primary antibody and anti-mouse osteocalcin primary antibody. The cells were then incubated at 4 °C overnight. Finally, an anti-rabbit secondary antibody FITC and anti-mouse secondary antibody rhodamine were added before the cells were analyzed using a Confocal Instrument Laser Scanning Microscopy (CLSM) at 488 and 543 nm.

Results: Quercetin increased the expressions of both osterix and osteocalcin in the osteoblast hFOB 1.19 cell line compared to the negative controls ($p<0.005$), with expression values of 57852*±3878.71 AU and 24161.75*±1498.65 AU, respectively.

Conclusion: Quercetin shows an anti-osteoporosis effect by increasing the expressions of both osterix and osteocalcin in osteoblast hFOB 1.19 cell line.

Keywords: Quercetin, Anti-osteoporosis, Osteoblast, hFOB 1.19 cell line, Osterix, Osteocalcin

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INTRODUCTION

Estrogen plays an important role in maintaining body homeostasis, such as maintaining bone mineral density and the balance between osteoblast and osteoclast activities [1]. Its deficiency that often occurs in postmenopausal women, has an impact on reduction in bone mineral density and damage to the micro-architecture of bone tissues [2-4]. Estrogen deficiency increases the release of cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), receptor activator nuclear factor kappa B ligand (RANKL), and macrophage colony-stimulating factor (MCSF). These result in increased bone resorption, as well as inhibition of bone formation [5], thereby leading to osteoporosis. Phytoestrogens are found in plants and they have a structure and function similar to estrogen [6, 7]. They have a function in maintaining homeostasis in the brain by binding to estrogen receptors, thereby making it a potential target for the treatment of diseases due to estrogen deficiency such as osteoporosis [7].

Quercetin (3,3′,4′,5,7 -pentahydroxyflavone) is a natural flavonoid compound that is found in vegetables and included in phytoestrogen compounds [8-11]. It has various health benefits such as anti-oxidant, anti-carcinogenic, neuroprotective, cardioprotective, anti-inflammatory, and anti-osteoporosis [1, 10]. *In vivo* quercetin increases the activity of bone and tissue cells, thereby inhibiting bone loss in ovariectomized and retinoic acid-induced mice [10, 12]. Also, it has been reported in an *in vitro* study that 10 µM quercetin can increase human osteoblast-like MG63 differentiation and proliferation, thereby down-regulating osteoclastogenesis of osteoclast precursor 2T-110 [8]. One of the cytokines, TNF-α, can inhibit the activation of bone formation transcription factor, osterix which plays a role in the differentiation and maturation of osteoblast [13, 14]. Osterix activation affects the regulation of the expression of bone formation proteins such as osteocalcin, collagen type I a1 (Col1a1), osteonectin, osteopontin, and bone sialoprotein (Bsp) [14-17].

In the present study, immunocytochemistry (ICC) was the method used because it is more selective against the test target. The cell is fluorescent due to fluorescence previously associated with secondary antibodies [18]. Generally, Confocal Instrument Laser Scanning Microscopy (CLSM) is used for analysis because the procedure has a high resolution from a shorter wavelength, better contrast quality compared to the conventional microscope, and does not affect the specimen [19]. The aim of this study was to investigate the effect of the administration of quercetin as a phytoestrogen on the activity of osterix and osteocalcin in hFOB 1.19 cells.

MATERIALS AND METHODS

Sources of cell line and chemicals

The hFOB 1.19 cell line (CRL-11372) was purchased from American Type Cell Culture (ATCC). Quercetin, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), paraformaldehyde (PFA), and phosphate-buffered saline (PBS) were obtained from Sigma-Aldrich, Merck SA, an affiliate of Merck KGaA, Darmstadt, Germany. Anti-rabbit osterix primary antibody and anti-mouse osteocalcin primary antibody was acquired from Abcam, 152 Grove Street Waltham, MA 02453 USA. Dulbecco's modified eagle's medium (DMEM), G418, penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Aretha Laboratory, Bandung, Indonesia. Tween-80, paraformaldehyde, anti-rabbit secondary antibody fluorescein isothiocyanate (FITC), and anti-mouse secondary antibody rhodamine were bought from the Central Laboratory of Life Science, Universitas Brawijaya, Malang, Indonesia.

Cell culture

The hFOB 1.19 cells were cultured in a 75 ml flask with a 5 ml complete medium (a mixture of DMEM, 1% penicillin-streptomycin, G418, 10% FBS) and incubated at 37 °C in a 5% CO_2 incubator until 80% confluence was achieved. The cells were then seeded on a 24-well microplate [6].

Osterix and osteocalcin measurement

The 10 µM quercetin [8, 20] sample was made by dissolving 10 mg of quercetin in a 1 ml complete medium. Induction of 10 ng/ml TNF-α was performed after the cells were cultured and reached 80% confluence on a 24-well microplate. After 24 h, the cells were rinsed with PBS and then treated with 10 µM quercetin for 48 h. The cells were then rinsed again

with PBS and fixed with 4% PFA, Triton X-100, BSA, a primary antibody for osterix, and osteocalcin for immunocytochemistry. The cells were incubated at 4 °C overnight. Then, the anti-rabbit secondary antibody FITC and anti-mouse secondary antibody rhodamine were added before being observed with CLSM (fluoview Olympus FV1000) at 488 nm and 543 nm. The immunofluorescence of markers was analyzed using Olympus Fluoview Ver.4.2a. software to determine the value of osterix and osteocalcin expression.

RESULTS

The measurement of osterix and osteocalcin expression was performed using the immunocytochemistry method and analyzed using a CLSM. This method makes it possible to view and evaluate the expression of proteins or other markers in cells with the principle of immunofluorescence [21].

The expression of osterix and osteocalcin after quercetin induction in hFOB 1.19 cell line was shown in fig. 1. Osterix expression was indicated by the fluorescence intensity in Arbittary Units (AU) which was measured after quercetin administration and compared to negative control. Osteocalcin expression was shown as the administration of quercetin showed an increase in osterix expression in the form of fluorescence intensity in AU compared to negative controls.

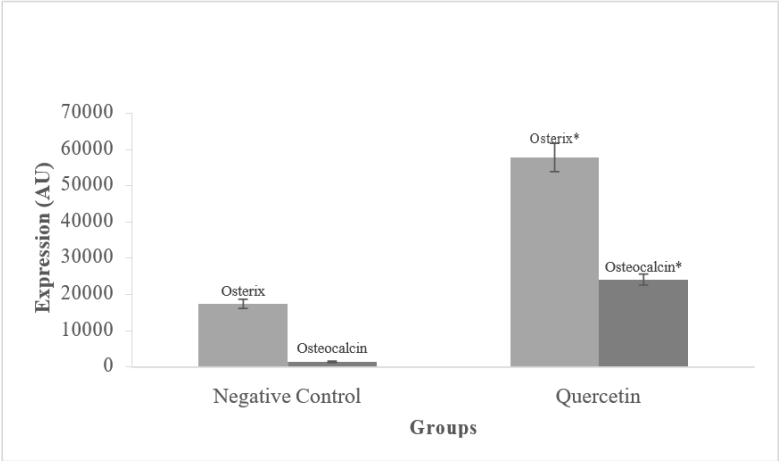

Fig. 1: Expression of Markers after Quercetin nduction in hFOB 1.19 Cell Line

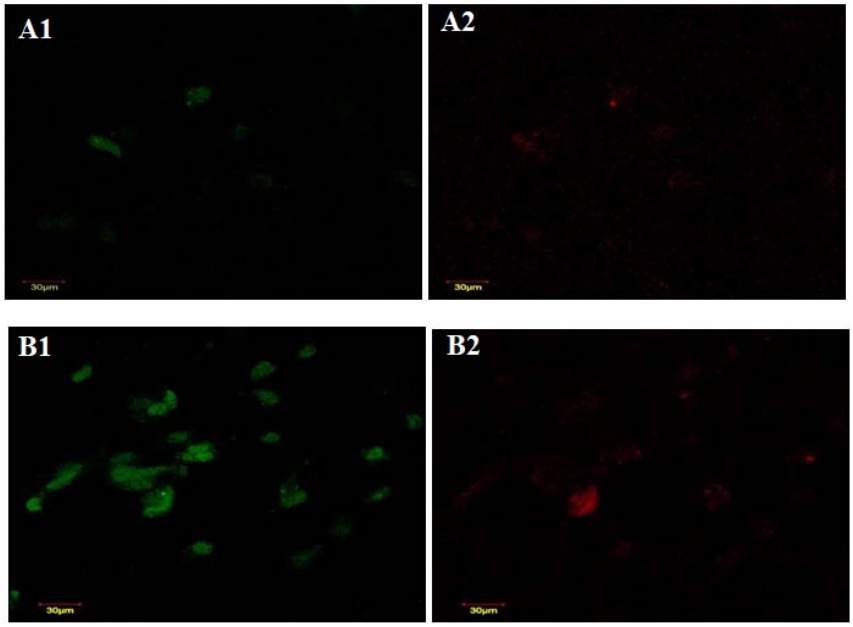

Fig. 2: The immunofluorescence visualization of markers in hFOB 1.19 cell line: (A) Negative Control, (B) Quercetin; (1) Osterix expression, (2) Osteocalcin expression

Table 1: Expression value of osterix and osteocalcin after quercetin induction in hFOB 1.19 cell line

Groups	Mean±SD (Arbitrary Unit (AU)	
	Osterix	Oteocalcin
Negative Control	17446.4±1298.86	1434.2±221.21
Quercetin	57852*±3878.71	24161.75*±1498.65

Note: The data were presented in mean+SD, n = 6

Visualization of marker expression in hFOB 1.19 cells is presented in fig. 1, while the results of the analysis of marker expression are highlighted in table 1. In fig. 1, the treatment of quercetin significantly increased osterix expression compared to the negative control with p=0.000. Also, a significant (p=0.000) expression of osteocalcin was observed compared to the negative control. The increase in osterix expression was indicated by an increase in the green color intensity of hFOB 1.19 cell line, while the increase in osteocalcin expression was indicated by an increase in the red color intensity of hFOB 1.19 cell line (fig. 2).

DISCUSSION

Postmenopausal women experience estrogen deficiency and are at risk of osteoporosis [22, 23]. There is an increase in proinflammatory cytokines such as TNF- α, IL-1, and IL-6 when estrogen levels are low [5, 24]. Osteoporosis in postmenopausal women has an increase in TNF-α, compared to those who do not have osteoporosis [25]. Inflammatory cytokines such as TNF-α and IL-6 play an important role in the state of bone resorption and bone remodeling disorders that lead to deterioration of bone architecture. The state of estrogen deficiency activates T-cells to produce TNF-α in excess and inhibit osteoblast activity, and inhibit bone component matrix such as osteocalcin and alkaline phosphatase [26-29].

TNF-α was administered to osteoblast cells hFOB 1.19 at a dose of 10 ng/ml for 24 h in this study to investigate the pathology of menopausal women [13, 29]. It was observed that TNF-α activates NF-kB, binds to RANKL, inhibits osteoblast cell maturation, and the expression of the transcription factor osterix and markers of bone formation processes such as osteocalcin [30]. The hFOB 1.19 cells are clonal human osteoprogenitor cells that are used as a model for the differentiation and maturation of osteoblasts [31]. Furthermore, hFOB 1.19 cells have an osteoblastic phenotype in terms of phenotypic markers and extracellular matrix mineralization, such as osteocalcin expression and others [32].

Quercetin is one of the phytoestrogen compounds that has an effect in increasing the expression of osterix and osteocalcin. Osterix is an osteoblast-specific transcription factor that is important for osteoblast differentiation and bone formation [15]. This increase in expression was caused by the role of quercetin as a phytoestrogen in increasing bone formation either through the estrogen receptor (dependent) and/or without the estrogen receptor (independent). The dependent pathway may be via binding to the core estrogen receptors (ER-α and/or ER-β) and membrane ER (GPR30 and ERX) present in the cell. The binding of estrogen and its receptors will activate the estrogen receptor. Activation of the estrogen receptor will bind to the estrogen response element (ERE) which can stimulate the transcription factors, Runx2 and Osterix as well as synthesize certain proteins such as osteocalcin [6, 33-35].

The measurable increase in osteocalcin expression after administration of quercetin in hFOB 1.19 cells showed an increase in a concomitant increase in the expression of the transcription factor of osterix. Increased activity of osterix stimulates maturation of osteoblast cells and stimulates increased expression of osteocalcin [36]. Increased osteocalcin expression can increase the bond between calcium and hydroxyapatite, which will occupy the bone matrix during mineralization, as well as bind residues and promote absorption of hydroxyapatite in the bone matrix, resulting in bone mineralization, which is the final stage of the bone formation process [37, 38]. Administration of quercetin to hFOB 1.19 cells showed an anti-osteoporosis effect with the mechanism of increasing bone formation activity. Quercetin administration causes an increase in the expressions of osterix and osteocalcin in hFOB 1.19 cells with estrogen deficiency.

Quercetin and its derivatives are potent natural osteogenic agents by multiple pharmacological activities including the function of antioxidant, anti-inflammatory, and estrogen-like effect *in vivo* and vitro studies [39, 40]. Administration of Quercetin to hFOB cells with estrogen deficiency has shown the ability to increase bone formation activity. This can be seen in estrogen-deficient conditioned cells given Quercetin showing increased expression of Osterix and Osteocalcin, which are important markers of bone formation activity. This research opens the opportunity for plants containing quercetin to be used as an anti-osteoporosis with bone formation mechanism.

CONCLUSION

The findings of this research suggest that quercetin shows anti-osteoporosis effects by increasing both osterix and osteocalcin expressions in osteoblast hFOB 1.19 cell line.

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AUTHOR CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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