ORIGINAL ARTICLE

Role of Melatonin in Down-regulation of Receptor Activator of Nuclear Factor kappa-B Ligand : Osteoprotegerin Ratio in Rat - Bone-Marrow Mesenchymal Stem Cells

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Abstract:

Background: Recent studies have reported that melatonin inhibits bone resorption through the regulation of Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL): Osteoprotegerin (OPG) ratio in osteoblast. However, the role of melatonin in osteoblast derived from MSCs is unclear. *Aim and Objectives:* To determine the down-regulation of RANKL:OPG ratio by melatonin supplementation in osteoblast culture in osteogenic medium derived from rat Bone-Marrow Mesenchymal Stem Cells (BM-MSCs). *Material and Methods:* This research was an experimental research conducted in a laboratory in vitro making use of rat BM-MSCs in osteogenic medium with or without melatonin for a duration of 21 days. Characteristics of MSCs were explored with the use of immunocytochemistry staining (CD45 and CD105). *Results:* After 15 days, mineralisation assay was carried out by means of Alizarin Red staining and at 21 days the RANKL and OPG levels were assessed with the use of sandwich ELISA. *Conclusion:* 150 nM or higher concentrations of melatonin could reduce RANKL levels; the supplementation of melatonin had no influence on OPG levels, and melatonin could reduce the RANKL:OPG ratio. The results of this study summarise that melatonin could reduce the RANKL:OPG ratio in the osteoblast culture originated from rat BM-MSCs.

Keywords: Melatonin, Osteoprotegerin, Receptor Activator of Nuclear Factor kappa-B Ligand, Bone Marrow Mesenchymal Stem Cells

Introduction:

Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) and Osteoprotegerin (OPG) are both essential regulators in the formation and activation of osteoclasts [1]. The increase in RANKL:OPG ratio correlates with an increase in the pathological bone resorption such as osteoporosis [2]. Osteoporosis in the field of dentistry has consequences on the condition of the teeth and oral cavity, and such consequences could include: an increase in alveolar bone resorption, decrease in mandibular cortical thickness, increase in risk of periodontal diseases, and tooth mobility up to tooth loss [3]. Mesenchymal Stem Cells (MSCs) are identical cells which play a part in the maintenance, repairing and regeneration of some body tissues, as well as bones [4]. Preceding studies have suggested that melatonin inhibits the process of bone resorption by decreasing the ratio of RANKL:OPG in osteoblasts as adult cells [5, 6]. However, it has not been well established as to whether melatonin possesses the same potential for decreasing the ratio of RANKL:OPG in osteoblasts culture derived from MSCs.

Melatonin is a neuroendocrine hormone and serves to improve bone health through the mechanism of bone remodeling as a target. Insufficient melatonin levels discovered in post-

menopausal women is known to disrupt bone remodeling and to increase the occurrence of osteoporosis [1]. This condition has received a reasonable level of attention from the field of dentistry due to the rising number of women with osteoporosis in Indonesia, which is directly proportional to the increase in life expectancy. Osteoporosis occurs not only at the lumbar spine, femur, and radius but also at the jaw bone [7]. Several studies have indicated that a fall in mandibular bone density came about in women experiencing osteoporosis [8, 9]. Another study also revealed a positive correlation between an alveolar bone height in the edentulous area and osteoporosis [10, 11]. It therefore calls for special attention from the field of dentistry.

Ostrowska *et al.* [12] indicated that down-regulation of plasma melatonin levels in post menopause women resulted in disruptions of bone metabolism and amplified the risk of osteoporosis. Melatonin enhanced healthy bones through the inhibition of bone resorption by regulating the RANKL:OPG ratio [5, 6]. This signifies that the relationship between the RANKL:OPG ratio and melatonin can be applied as a basis to conduct a research on the use of melatonin as an alternative therapy for diseases which heightens bone resorption.

This study was conducted to ascertain the down-regulation of RANKL:OPG ratio in osteoblast cells culture obtained from rat BM-MSCs which have been exposed to melatonin with various doses of osteogenic medium.

Material and Methods:

This study was conducted with the approval of the ethical committee of Faculty of Dental Medicine, Universitas Airlangga with certificate number 25/KKEPK.FKG/II/2016.

Isolation, Culture, and Expansion of Rat BM-MSCs

Rat BM-MSCs were extracted from two femurs of a 6 weeks-old male Wistar rat of the Albino strain. The rat was put into an anesthesia chamber and was sacrificed using ketamine and diazepam. The femurs were collected under sterile conditions, and then all the femurs were cut at both ends. The bone marrow from each bone was collected by flushing the interior of the bone with 3 ml minimum essential Medium-α Modification (αMEM). After filtering, the cells were centrifuged at 1600 rpm for 10 min and the cell pellets were resuspended in 6 ml α-MEM. The purified cells were then plated in a 5 cm petridish and finally expanded in growth medium containing αMEM, 10% fetal bovine serum, 100 units/ml of penicillin G, and 100 µg/ml of streptomycin. The MSCs were incubated at a temperature of 37°C in a humidified atmosphere of 5% CO_2. Non-adherent cells were removed by changing culture media after 24 h. The complete medium was replaced every 3 days. Cell viability was verified by a continuous cell division, and the cells were subcultured using 3 ml of 0.05% trypsin/EDTA when cells reached about 80% confluence [13]. Cells of fifth passage were utilised for the purpose of studies.

Characterisation of Rat BM-MSCs

For the identification of cell characteristics after the growth in culture, identical MSCs were subjected to immunocytochemistry staining. Two surface markers of BM-MSCs at passage 5 were assayed. Samples were trypsinised, embedded in -20°C acetone for duration of 10 mins, blocked using fetal calf serum 1%, and left to dry. After further Phosphate Buffer Solution (PBS) washes, cells were incubated for 45mins at 37°C with the primary antibodies (CD45). After washing the cells with PBS, cells were incubated with secondary

antibodies (CD105) for another 45 min at 37°C. Incubations were followed by washing with PBS and FITC treatment labeled with conjugate Fab IgG. The mounted cells were scrutinised under a fluorescence microscope with a green color filter [13].

Induction of Osteogenic Differentiation

Cells collected from passage 5 were then seeded onto 24-well plates in Osteogenic (OS) medium, which contains growth medium supplemented with 0.17 mM ascorbate, 10 mM β-glycerophosphate, and 0.1 mM dexamethasone [14]. Cells were preserved with the addition of fresh osteogenic medium every 3 days for 21 successive days [15], with a final volume of 1ml in each well.

Treatment Groups

Eight treatment groups [OS+ M-, OS+ M+ (25 nM, 50 nM, 100 nM, 150 nM, 500 nM, 1000 nM, and 5000 nM)] were put to use all through this study. The groups were exposed to OS+ in the absence (OS+ M-) or presence (OS+ M+) of melatonin to stimulate osteoblasts differentiation. These studies were conducted for a total of 21 days.

Alizarin Red Staining

After osteogenic induction for 15 days, mineral deposition was evaluated by staining with Alizarin red. Briefly, excess medium on cells were shaken off with PBS, embedded in 10% (v/v) formaldehyde, and then left to dry completely. After 15 min, cells were stained with 2% alizarin red solution (pH 4.1-4.3), incubated for 20mins at room temperature, and then followed by rinsing with distilled water [15]. The plate was visualised with the use of a light microscope.

Determination of OPG and RANKL Expression

On day 21, the concentration of OPG and RANKL were assayed using a commercial ELISA (Elabscience) in accordance with the manufacturer's protocol. For the establishment of OPG expression, cell culture supernatant was harvested from cultured cells, centrifuged to remove debris, and stored at −20°C pending the time of use. Moreover, for the establishment of RANKL expression, cell was harvested from each well on the 21st day, supplemented with RIPA buffer, and stored at −20°C pending the time of use [16]. The OPG and RANKL concentrations were ascertained by comparison to a standard curve.

Statistical Analysis

All experiments were repeatedly conducted for a minimum of six times. The results are recorded as the mean ± SD. All statistical analyses were performed using SPSS 17.0. Shapiro-Wilk tests and Levene test were employed for analysing the distribution and homogeneity of data. Non-parametric statistical analyses were conducted by Kruskal-Wallis test due to the variables did not showing a regular distribution and/or homogeneity, and this was then followed by Mann-Whitney test. $p < 0.05$ was considered to be significant.

Results:

Rat BM-MSCs Culture

Bone marrow was extracted from the femurs of a rat and plated into a 5 cm culture dish. Non-adherent cells were cautiously removed after 24 hours and 1.5 ml fresh medium was replaced. The adherent cells were then washed with PBS, and fresh medium was added time after time with an interval of 3 days. On day 1 after isolation, majority of the cells were hematopoietic cells and MSCs were hardly seen. After 5 days of initiation culture, it was observed a dramatic decrease in hematopoietic stem cell lineages and some spindle-shaped/fibroblast-like cells appeared.

During the second week of culture, the number of spindle-shaped/fibroblast-like cells became more confluent, grew out from a dense cell nodule, and reached about 90% confluence (Fig. 1).

Confirmation of Phenotype and Osteogenic Differentiation Assay of Rat BM-MSCs

The fifth passage cells were analyzed for cell surface antigens. Figure 2 illustrated that the established cells were strongly positive for CD105 and less positive for CD45. The osteogenic differentiation capacities of BM-MSCs were obtained with the use of Alizarin red staining. And as illustrated in Figure 3, the cells were stained positively after 15 days for extracellular mineralisation in all groups.

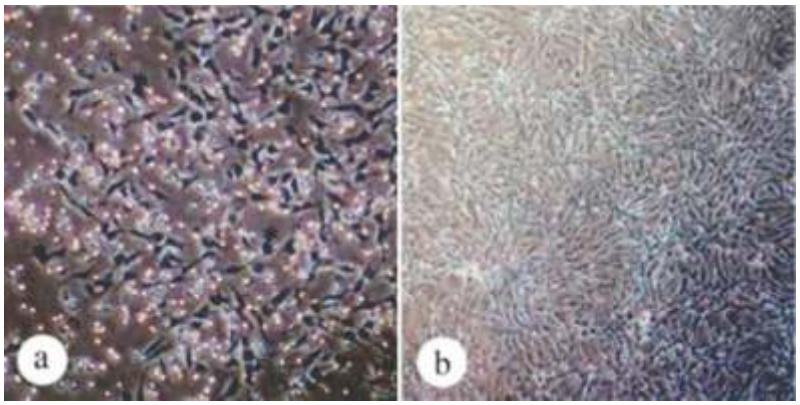

Fig. 1: Representative of Rat BM-MSCs Culture. a. After 5 Days, Most of MSCs were Adherent to Dish, showing Spindle-shape or Fibroblast-like Cell Morphology. b MSC showed More Dense and has Reached Confluence.

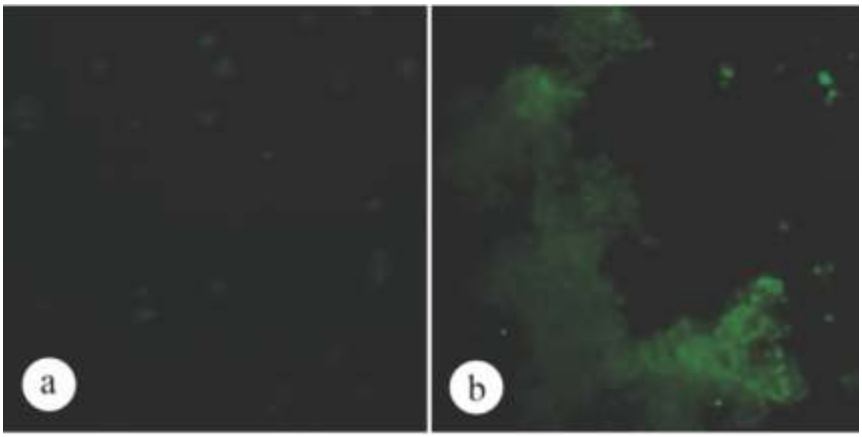

Fig. 2: Representative Immunocytochemistry Staining of rat BM-MSCs. a Rat BM-MSCs Less Expressed CD45. b Rat BM-MSCs Strongly Expressed CD105.

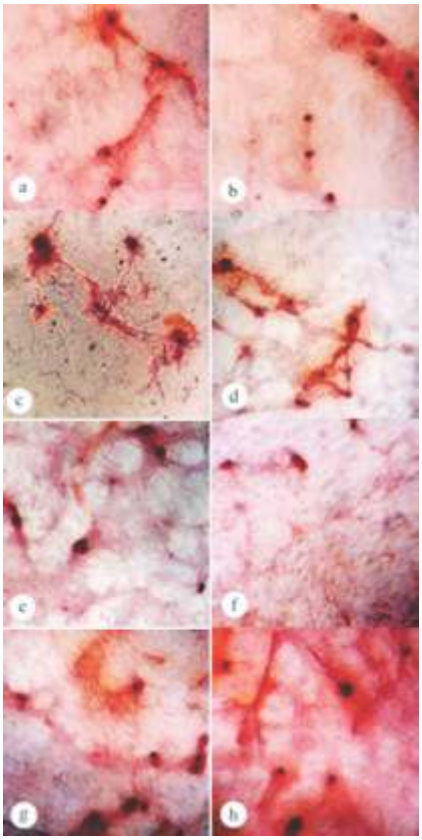

Fig. 3: Representative Alizarin Red Staining, Bright Red Appearance Found in All Groups indicating the Presence of Extracellular Mineral Deposit. in Presence of Melatonin 5000 nM (a) 1000 nM (b), 500 nM (c), 150 nM (d), 100 nM (e), 50 nM (f), 25 nM (g), and Control (h).

Protein Expression Analysis

At 3 weeks, we examined the effects of melatonin has on RANKL and OPG expression in osteoblast obtained from rat BM-MSCs using ELISA. The result demonstrated that the expression of RANKL was relatively lesser when compared with controls ($p < 0.05$). Melatonin decreased RANKL level in a dose-dependent manner and that significant reduction was observed in 150 nM or higher concentrations of melatonin. Melatonin supplementation had no influence on OPG expression. There was no significant difference perceived between the groups ($p > 0.05$).

RANKL:OPG Ratio

RANKL:OPG ratios were significantly lower when compared to those with controls ($p < 0.05$). Melatonin reduced RANKL:OPG ratios in a dose-dependent manner and its lowest doses (25 nM) or higher concentrations markedly reduced RANKL:OPG ratios.

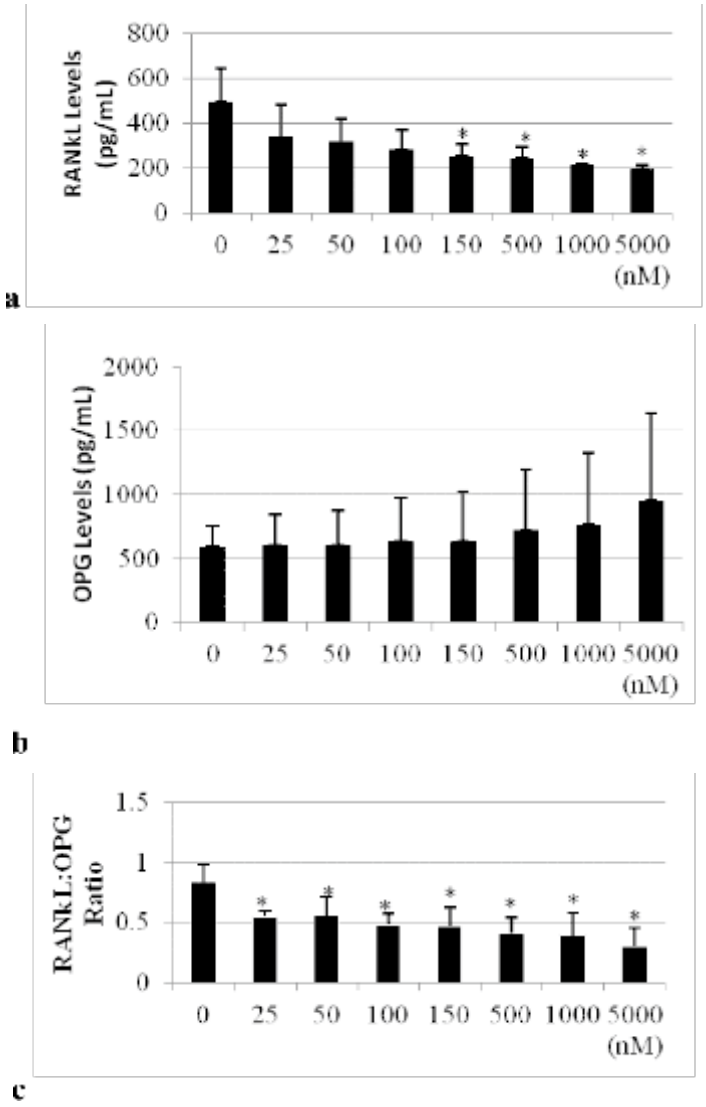

Fig. 4: Effect of Melatonin in RANkL Levels, OPG Levels, and RANkL:OPG Ratio in Osteoblast Cell Culture Derived from Rat BM-MSCs Exposed to Osteogenic Medium. Experiments were Conducted Repeatedly at least 6 times, and Data were Presented as mean ± SD. Rat BM-MSCs were Exposed to Various Concentrations of Melatonin for 21 Days and then RANkL and OPG levels were Quantified. A RANkL Inhibition by Melatonin. The RANkL Levels when the Rat BM-MSCs were Not Exposed to Melatonin (0nM) were Employed as Control. *p < 0.05. b OPG Levels Induction by Melatonin. RANkL Levels When the Rat BM-MSCs were Not Exposed to Melatonin (0nM) were Employed as Control. *p < 0.05. c RANkL:OPG Ratio Inhibition by Melatonin. The RANkL:OPG ratio When the Rat BM-MSCs were Not Exposed to Melatonin (0nM) was Employed as Control. *p < 0.05.

Discussion:

In this study, we have isolated and characterized a population of rat BM-MSCs. The isolation protocols made use of the Laboratory of Stem Cell, Institute of Tropical Disease, Airlangga University protocol. The morphology of the isolated cells was consistent with the characteristics of MSCs which have been stated by various studies in the literature. They were spindle-shaped/fibroblast-like cells adhered to the petridish. It was conducted a characterization process using immunocyto-chemistry. Cultured rat BM-MSCs are uniformly and solidly positive for CD105, CD90, and CD73, regardless of their passage or time spent in culture, and on the other hand, failed to express CD45, CD31, and CD 34 [17]. CD105 is MSCs marker, and potentially plays a role in TGF-beta signaling in the course of MSC chondrogenic differentiation; on the contrary, CD45 is hematopoetic markers [18]. The obtained result signified that CD105 was highly expressed. It was also considerably similar to those of previous studies which have demonstrated rat BM-MSC characterization. They found positive CD73, CD90 and CD105 expression on passage one [19]. Preceding studies also established that positive expression of CD73, CD105, and Stro-1 reached the highest expression on passages 3-6 [20]. Wang *et al.* [21] also derived at a similar result, depicting positive expression of CD29, CD105, CD166, and VLA-4 on the fourth passage. It also indicated positive expression of CD45, though it seemed as weak. One of the criteria put in consideration for the characterization of MSC is the negative expression of CD45 [22]. This condition may arise as a result of the immunocytochemistry technique is not being a quantitative test, which means it is not capable of accurately determining the amount of CD45 [23]. Passage also affects the expression of markers of MSCs. Study conducted by Harting *et al.* [20] discovered positive expressions of CD11b and CD45 on passage 3, which was decreasing with the number of passages [20]. Another explanation that may arise is the possibility of MSC colonies for not being completely separated from hematopoietic stem cells [23].

Alizarin Red staining was carried out to detect osteogenic differentiation in cultures of rat BM-MSCs. Osteogenic differentiation is characterized by extracellular mineralisation indicated by a red color on Alizarin Red inspection. Stained osteoblasts appear bright red in colour, whereas undifferentiated MSCs do not [24]. In the course of this study, all groups displayed a noticeable amount of red-stained cell clusters, indicating that the rat BM-MSCs collected were differentiated towards the osteoblast lineage.

In the current study, we tested the hypothesis that melatonin affects the RANκL:OPG ratio on osteoblast generated from rat BM-MSCs. Under regular physiologic conditions, there is a balance between bone resorption and bone formation. This balance enhances bone homeostasis. In certain inflammatory bone conditions, the balance is altered to enhance the occurrence of excessive bone resorption, as that observed in osteoporosis. Accordingly, a relative decrease in the concentrations of OPG or an increase in RANκL expression may result in a net increase in RANκL and pathologic bone resorption, which is also known as an increase in the RANκL:OPG ratio [2, 25]. RANκL is a type II transmembrane protein located on the surface of expressing cells as a proteolytically released soluble form. Its expression through osteoblasts coordinates bone

remodelling by means of stimulating bone resorption via local osteoclasts, which in turn stimulate bone synthesis by closely adjacent osteoblasts through a process known as 'coupling' [25, 26]. OPG therefore serves as a decoy receptor by blocking RANKL binding to its cellular receptor RANK. Expression of RANKL and OPG is therefore coordinated to regulate bone resorption and density both positively and negatively by controlling the activation state of RANK on osteoclasts [25].

In the current study, RANKL:OPG ratios were relatively lower when compared with controls. They were reduced by melatonin in a dose-dependent manner and this evident reduction was a result of either the lowest doses of melatonin at 25 nM or higher concentrations. It was also caused by melatonin supplementation reducing the RANKL level in a dose-dependent manner and that significant reduction was observed with 150 nM or higher concentrations of melatonin. Koyama *et al*. [6] stated that melatonin addition for 5 hours in MC3T3-E1 cells enhanced bone mineral density by inhibiting bone resorption trough down-regulation of RANKL transcript level in a dose-dependent manner with significant reduction in 10 μM or higher concentrations of melatonin. A previous study by Histing *et al*. [5] similarly indicated that the supplementation of melatonin 50 mg/kg body weight i.p. daily in the course of the entire 2 weeks in mice could impair fracture healing by suppressing bone resorption through downregulation of RANKL-mediated osteoclast activation

Melatonin could downregulate RANKL levels in osteoblast derived rat BM-MSCs which have been exposed to osteogenic medium as a result of Wnt signaling activation. Wnt signaling has potential roles to play in bone remodeling in both physiological and pathological conditions. It suppresses bone resorption by regulating RANKL levels through the β-catenin dependent canonical pathway [27]. In this research, the activation of Wnt signalling with 20mM LiCl exposure for 24 hours led to the downregulation of RANKL, mRNA and protein expressions, as well as the overexpression of full length β-catenin. Melatonin activated Wnt signalling [28]. Other studies showed that 50nM melatonin supplementation for 30-240 minutes activated Wnt 5a/b and β-catenin, while it attenuated phosphorylation of glycogen synthase kinase-3β (GSK-3β) in MC3T3-E1 cells [29].

The addition of melatonin on MSCs cultured exposed to osteogenic medium, binds with MT2R to directly activate and phosphorylate MAPK (ERK 1/2) [1, 29-30]. Up-regulation of ERK 1/2 activity increased transcriptional activity of Runx2 and OSX, in order for it to induce MSCs differentiation into mature osteoblasts. Supplementation of melatonin has been found to activate Wnt pathway which leads to an increase in the transcriptional activity of Runx2 and OPG gene, as well as an inhibition of RANKL gene transcriptional activity [27]. An increased activity of osteoblastic specific gene through ERK 1/2 pathway and Wnt pathway would trigger the differentiation of MSCs into mature osteoblasts cells, which secrete OPG in higher quantities and RANKL in relatively lower quantities. The results indicated a fall in the ratio of RANKL:OPG obtained in all groups, with diminished levels of RANKL, but there were no differences in OPG levels in all groups due to very low melatonin doses.

Conclusion:

About 150 nM or higher concentrations of melatonin could reduce RANKL levels, its supplementation had no effect on OPG levels, and it is capable of reducing the RANKL:OPG ratio. The results of this study summarise that melatonin is capable of reducing the RANKL:OPG ratio in osteoblast culture obtained from rat BM-MSCs.

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