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Original article

Melatonin and astaxanthin modulate RANKL-induced TRAP activity in RAW264.7 cells in an opposite fashion

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Abstract

***Objective*:** Melatonin ($C_{13}H_{16}N_2O_2$) and astaxanthin ($C_{40}H_{52}O_4$) are two important biomolecules that show various effects *in vitro* and *in vivo*. Melatonin secretion and bone strength fall progressively as we age. Astaxanthin is a natural carotenoid that protects against severe environmental conditions (*e.g.* UV radiation, free radicals). Therefore, we investigated the *in vitro* effect of melatonin and astaxanthin on RANKL-induced osteoclastogenesis of RAW264.7 cell.

***Methods*:** We used murine monocyte/macrophage RAW264.7 along with 100 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) as a model for osteoclastogenesis. Melatonin and astaxanthin (AstaReal) were firstly dissolved into dimethyl sulfoxide (DMSO) and then diluted, as required. To determine osteoclastogenesis, Tartrate-resistant acid phosphatase (TRAP) activity was measured and cells were stained for counting TRAP positive multinucleated cells. Cell counting kit-8 (CCK-8) and lactate dehydrogenase (LDH) assay were used to check cytotoxicity.

***Results*:** Melatonin at a dose of 10 μg/mL significantly stimulated RANKL-induced TRAP activity without altering TRAP positive multinucleated cell number; but at a lower dose, it shows no effect. On the other hand, astaxanthin significantly downregulated TRAP activity without altering TRAP positive multinucleated cell number. When we used melatonin and astaxanthin together, they countered each other's effect. None of the experimental conditions causes cell death.

***Conclusion*:** Melatonin and astaxanthin significantly modulated RANKL-induced TRAP activity in RAW264.7 cells in positive and negative ways, respectively.

***KEY WORDS*:** Osteoclastogenesis, RANKL, melatonin, astaxanthin, RAW264.7 cells.

Introduction

Bone remodeling is a lifelong process primarily maintained by bone cells, bone-resorbing osteoclast (differentiated from hematopoietic stem cells: HSCs) and bone forming osteoblast cells (differentiated from mesenchymal stem cells: MSCs). They also play a crucial role in maintaining blood calcium homeostasis by making a balance with bone metabolism (bone resorption and bone formation). Osteoclasts initiate the process by resorbing discrete parts of old bone and later activate osteoblast differentiation and activation, migrate to the site of action, and there replace the old bone with newly produced packets of a proteinaceous matrix. Any imbalance in the regulation of bone remodeling can lead to several metabolic bone diseases like osteoporosis, osteopenia, pre-menopausal bone loss, Paget's disease, *etc.*[1-3].

Every three seconds, there occurs one osteoporosis-related bone fracture worldwide. More than 200 million postmenopausal women have osteoporosis and may experience hip or vertebral fracture that could lead to death[4]. Adequate bone quality and density are required to carry body weight and resist fracture. Bone quality and density are maintained by adequate resorption by osteoclast cells followed by sufficient bone formation by osteoblast cells. Therefore, proper differentiation, maturation, and activation of the bone cells are of great importance for maintaining appropriate bone density and strength[5].

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Melatonin (N-acetyl-5-methoxytryptamine) is a natural mammalian hormone, mainly found in the pineal gland. It is also present in the retina, bone marrow, lymphocytes, and even in foods such as oats. It exhibited a potential anti-inflammatory and antioxidant role; it also has regulatory roles in immunomodulation and cardiovascular functions. Further, it has been suggested to have anti-aging properties. In humans, melatonin secretion declines progressively with age and after menopause; also bone density and strength decline with age and after menopause [6, 7]. Therefore, we hypothesized that being in bone marrow, melatonin may play some crucial role in bone remodeling. Melatonin showed osteoblast-inducing effects on human adipose-derived MSCs (mesenchymal stem cell) and inhibited osteoclastogenesis in layered coculture system of human MSC and human peripheral blood monocytes (PBMCs) by increasing OPG: RANKL ratios by inhibiting RANKL secretion [8]. In this present study, we focused on the direct effect of melatonin on RANKL-induced *in vitro* osteoclastogenesis using RAW264.7 cells. Also, our laboratory has been investigating the astaxanthin-containing supplement and revealed the effects on oxidative stress in the human pilot study [9] and on intestinal microflora in high-fat-diet-fed mice [10]. In this present study, we checked the effect of melatonin and astaxanthin (***Fig. 1***) on RANKL-induced osteoclastogenesis in RAW264.7 cell.

Materials and methods

Melatonin was obtained from Sigma-Aldrich (St. Louis, MO). As astaxanthin, an equivalent of the commercially available "AstaReal ACT^{R}" was provided by AstaReal Co., Ltd. (Minato-ku, Tokyo, Japan) and used. Every two capsules contained 6.05 kcal, 0.27 g of protein, 0.53 g of lipid, 0.05 g of carbohydrate, and 0.1 mg of Na. The test product was made from olive oil, gelatin, *Haematococcus pluvialis* (12 mg as astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) per 2 capsules), tocotrienol, glycerin, glycerol ester, beeswax, and L-ascorbic acid 2-glucoside [9]. Both test products were dissolved into dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) and 2 µL/mL were used in all cell culture experiments to avoid DMSO cytotoxicity. An equal volume of DMSO was also used as vehicle control.

Cell culture and reagents

The murine monocyte/macrophage RAW264.7 (ATCC® TIB-71TM) cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan), penicillin 100 units/mL, streptomycin 100 µg/mL and amphotericin B 25 µg/mL (Gibco, El Paso, TX) at 37 °C under the condition of 5% CO_2 [11-13]. RAW264.7 subculture passage 3 to 6 were used for all experiments.

In vitro osteoclastogenesis

RAW264.7 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h for cell attachment. The media were then changed with αMEM (Gibco, El Paso, TX) with the mentioned concentration of melatonin or astaxanthin, 100 ng/mL recombinant mouse RANK Ligand (rmRANKL, R&D systems, Minneapolis, MN) with FBS and antibiotics [11-13]. After 3 days, the medium was renewed. After 5 days of cultures, cells were observed by multiple assays as mentioned.

TRAP staining

Cells were fixed in a 10% formalin neutral buffer solution and stained using a TRAP staining kit (Cosmo Bio Co., LTD., Tokyo, Japan) according to the manufacturer's instruction. TRAP is a widely-used marker of osteoclast maturation and function [11]. Multinucleated cells having ≥ 4 nuclei were counted under a light microscope as an osteoclast cell.

TRAP activity

Treated cells were fixed after 5 days of treatment using cell fixation buffer (Acetone: Ethanol = 1 : 1) and then fixed cells were used to measure TRAP activity with a TRAP solution kit (Oriental Yeast Co., Tokyo, Japan) according to the manufacturer's instruction. Colorimetric absorbance was taken at 405 nm using a Varioscan® Flash (Thermo Scientific, Waltham, MA) microplate reader [11-13].

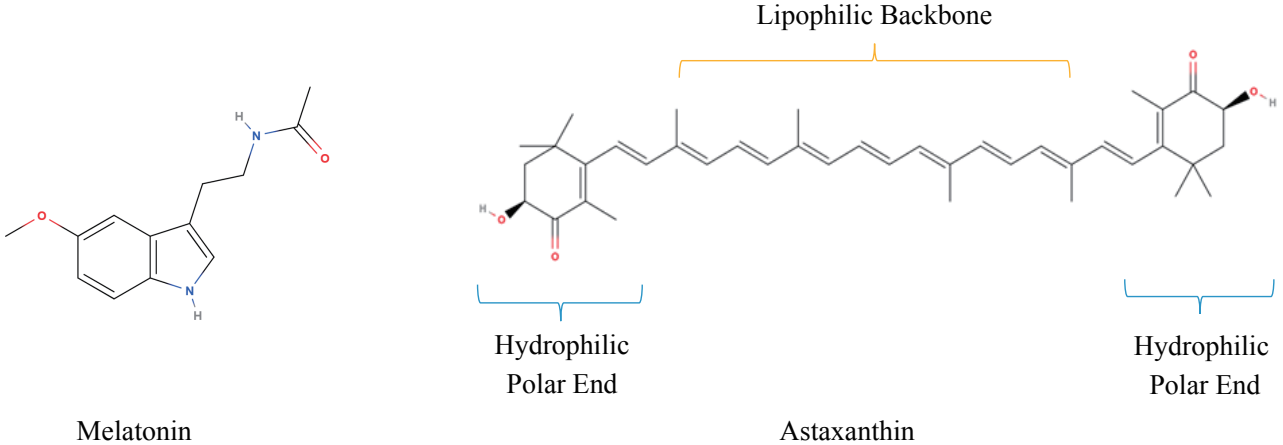

Fig. 1. **The structure of melatonin and astaxanthin.**

Evaluation of cell viability

Cell viability was evaluated using CCK-8 (Dojindo, Kumamoto, Japan). RAW264.7 cells were seeded on 96-well plates at the mentioned density and were treated as regular experiments described in the results section. After 5 days, a 10% volume of CCK-8 solution was added to the culture medium and the cells were incubated for 1 h. Absorbance at 450 nm was then measured as previously described using a Varioscan® Flash microplate reader [11-13].

To check cytotoxic cell death, an LDH assay was performed [12, 13]. RAW264.7 cells were seeded and treated as regular experiments. Then, cultured media (50 µL) from each well was collected and used to determine cell cytotoxicity (LDH secretion into media) in RAW264.7 cells. After incubating with assay solution and red color development, colorimetric absorbance was measured at 490 nm using a Varioscan® Flash microplate reader.

Statistical analysis

Data were expressed as means ± standard error of the mean (SEM). All statistical analysis was performed using the Tukey-Kramer test for intergroup comparison in all experiments. Differences were considered significant at a significance level of 5%.

Results

Melatonin and astaxanthin modulated RANKL-induced TRAP activity in RAW264.7 cells in the opposite fashion:

Melatonin and astaxanthin were dissolved into DMSO. Therefore, an equal volume of DMSO was used along with RANKL as vehicle control. At a lower concentration (0.1 µg/mL), melatonin has no effect on RANKL-induced TRAP activity. However, at a higher concentration (10 µg/mL), it significantly induced TRAP activity. Whereas, astaxanthins slightly reduced TRAP activity at lower concentration (5 µg/mL), and significantly reduced TRAP activity at higher concentration (50 µg/mL, ***Fig. 2-a***).

None of the experimental conditions caused cell death (***Fig. 2-b***). In the absence of RANKL, melatonin fails to induce TRAP activity (***Fig. 2-c***) showing that this effect is RANKL-dependent.

Melatonin and astaxanthin controlled multinucleated osteoclast cell size (***Fig. 3-a***) instead of controlling multinucleated osteoclast cell numbers (***Fig. 3-b, c***).

Both melatonin and astaxanthin counteracted each other's effect:

TRAP activity was induced by melatonin and reduced by astaxanthin in our experimental conditions. Therefore, we checked whether stimulatory melatonin and inhibitory astaxanthin could neutralize each other's effects or not. Melatonin-stimulated TRAP activity was highest, and astaxanthin inhibited to the lowest. When melatonin and astaxanthin were both used together, TRAP activity was between melatonin and the astaxanthin only group showing that they can counterbalance each other's effect without causing cell death (***Fig. 4***).

Discussion

In the present study, we analyzed the effect of melatonin and astaxanthin on RANKL-induced osteoclastogenesis of RAW264.7 cells and found both have the opposite effect. RANKL-induced TRAP activity was significantly induced by melatonin (***Fig. 2-a***) without causing cell death (***Fig. 2-b***). Melatonin was previously reported to inhibit osteoclastogenesis by increasing OPG:RANKL ratios by inhibiting RANKL secretion in layered cocultures of human MSCs and human PBMCs [8]. Here we examined the direct effect of melatonin on RANKL-induced osteoclastogenesis in RAW264.7 cells and found that it induced osteoclastogenesis. In the absence of RANKL, melatonin itself did not induce osteoclastogenesis (***Fig. 2-c***) showing that melatonin is not osteoclastogenic, but it can stimulate RANKL-induced osteoclastogenesis in RAW264.7 cells. Astaxanthin exhibited an inhibitory effect on RANKL-induced osteoclastogenic TRAP activity (***Fig. 2-a***). The stimulatory effect of melatonin and the inhibitory effect of astaxanthin was found to be controlled by osteoclast cell size (***Fig. 3-a***) instead of cell number (***Fig. 3-b, c***) as they stimulated and inhibited giant osteoclast cell production, respectively without changing osteoclast cell numbers. Melatonin may stimulate the fusion between osteoclast cells (multinucleated Cells ≥10 nuclei) and produced giant osteoclast cells. On the other hand, astaxanthin may inhibit the fusion between osteoclast cells (multinucleated Cells ≥10 nuclei) and thus produced relatively smaller osteoclast cells (***Fig. 3-a***).

The stimulatory effect of melatonin and the inhibitory effect of astaxanthin counteract each other's effect, suggesting that this effect is not a fluke and that both of their effects are real and could be modulated. However, melatonin's osteoclastogenesis inhibitory effects were facilitated through MT2 melatonin receptors, MEK1/2, and MEK5 in layered cocultures of human MSCs and human PBMCs by inhibiting RANKL secretion by MSCs [8]. In the present study, we found that melatonin stimulates RANKL-induced osteoclastogenesis. Together, melatonin is a multifunctional compound that behaves differently with different cell types. Being in bone marrow, melatonin is modulating bone remodeling. In elderly men and women after menopause, melatonin secretion falls, followed by a decline of bone mass and quality. By giving them adequate melatonin, we can expect to reverse the bone turnover and thereby reverse both bone mass and quality loss.

On the other hand, astaxanthin showed the opposite effect of melatonin, and their combined use confirmed both effects are real (***Fig. 4***). Astaxanthin reduced osteoclastogenesis may be followed by a reduction of bone resorption. Thus, astaxanthin may help to prevent high bone-metabolic rotation osteoporosis in elderly men and menopausal women.

Therefore, melatonin and astaxanthin have the potential to be used in different bone-related diseases where bone formation or resorption need to be controlled. A detailed study is required to reveal the molecular mechanism of melatonin stimulation and astaxanthin inhibition of RANKL-induced osteoclastogenesis in RAW264.7 cells.

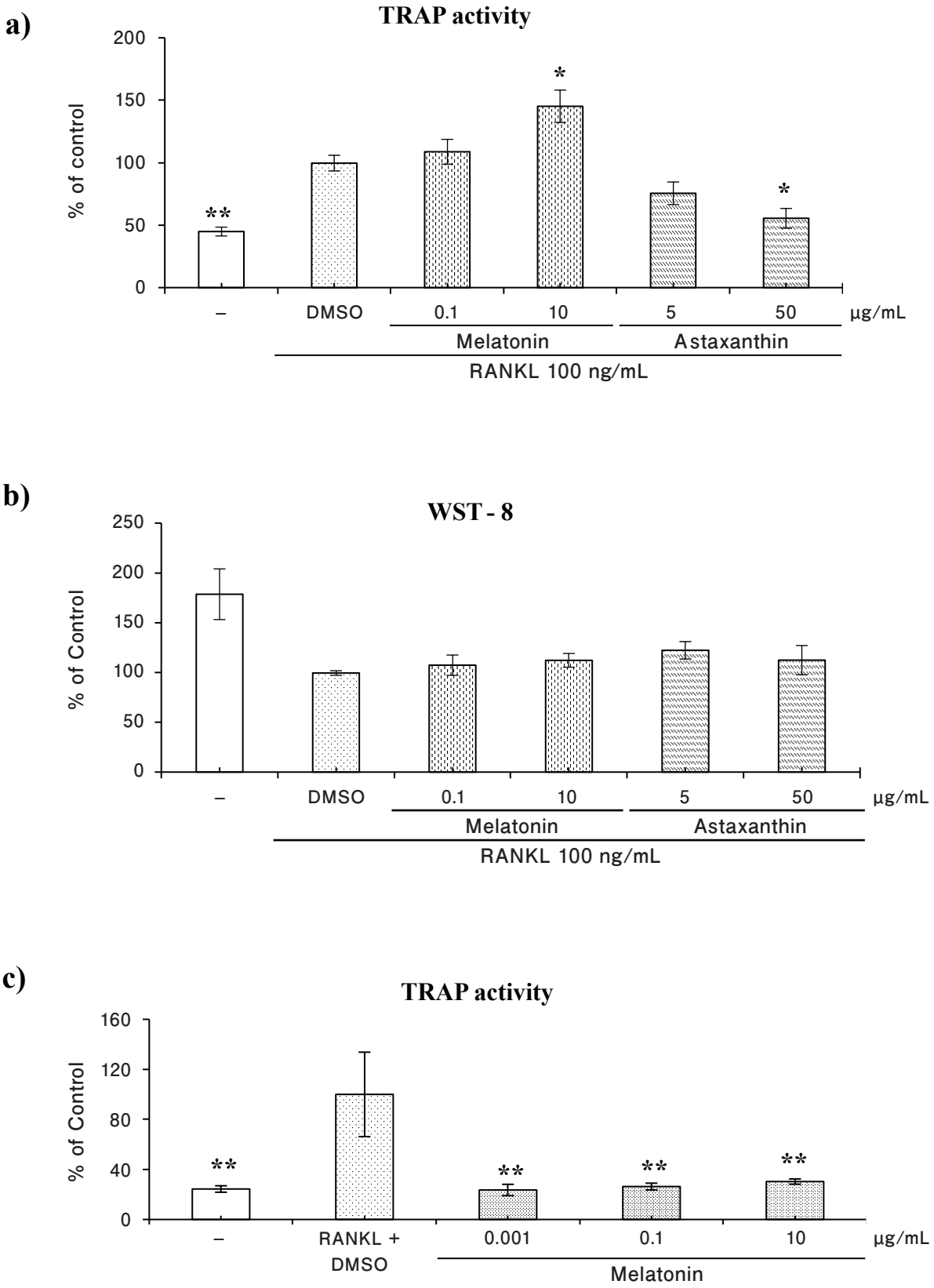

Fig. 2. **Effect of melatonin and astaxanthin on TRAP activity and cell viability.**

RAW264.7 cells were treated with αMEM containing 10 % FBS, 100 ng/mL RANKL with or without differing doses of melatonin and astaxanthin for 5 days. **a)** TRAP activity, **b)** WST-8 assay, **c)** Effect of melatonin alone (in the absence of RANKL) on TRAP activity. All data are shown as means ± SEM, n = 6. *$p < 0.05$, **$p < 0.01$, Tukey-Kramer test. TRAP, tartrate resistant acid phosphatase; RANKL, receptor activator of nuclear factor kappa-B ligand; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum, WST, water-soluble tetrazolium-salt; SEM, standard error mean.

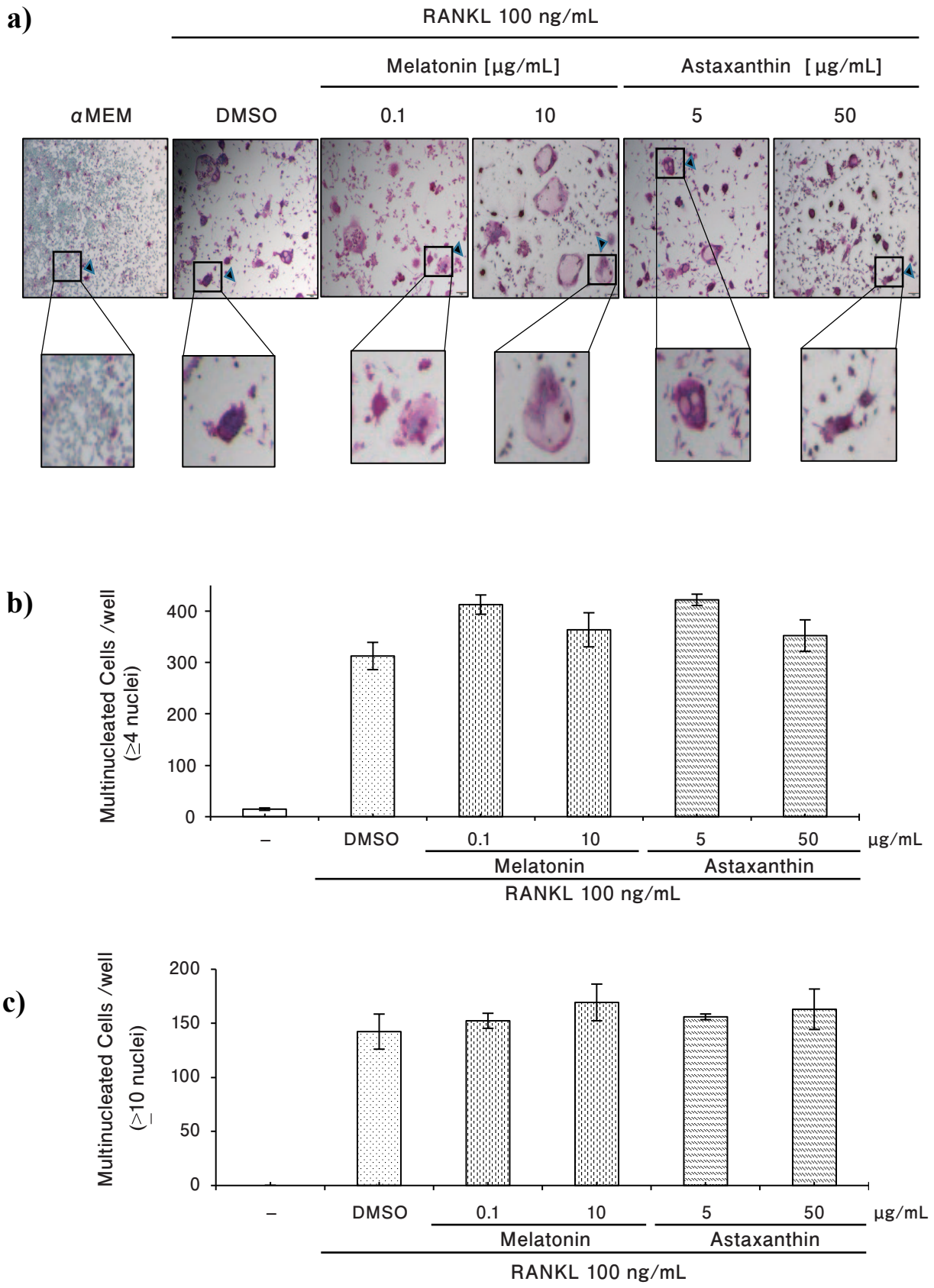

Fig. 3. **Effect of melatonin and astaxanthin on TRAP positive multinucleated cell formation.**

RAW264.7 cells were treated with αMEM containing 10 % FBS, 100 ng/mL RANKL with or without differing doses of melatonin and astaxanthin for 5 days. **a)** TRAP staining, **b)** Multinucleated cells having ≥4 nuclei, **c)** Multinucleated cells having ≥10 nuclei. All data are shown as means ± SEM, n = 6. *p < 0.05, **p < 0.01, Tukey-Kramer test. TRAP, tartrate resistant acid phosphatase; RANKL, receptor activator of nuclear factor kappa-B ligand; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum, WST, water-soluble tetrazolium-salt; SEM, standard error mean.

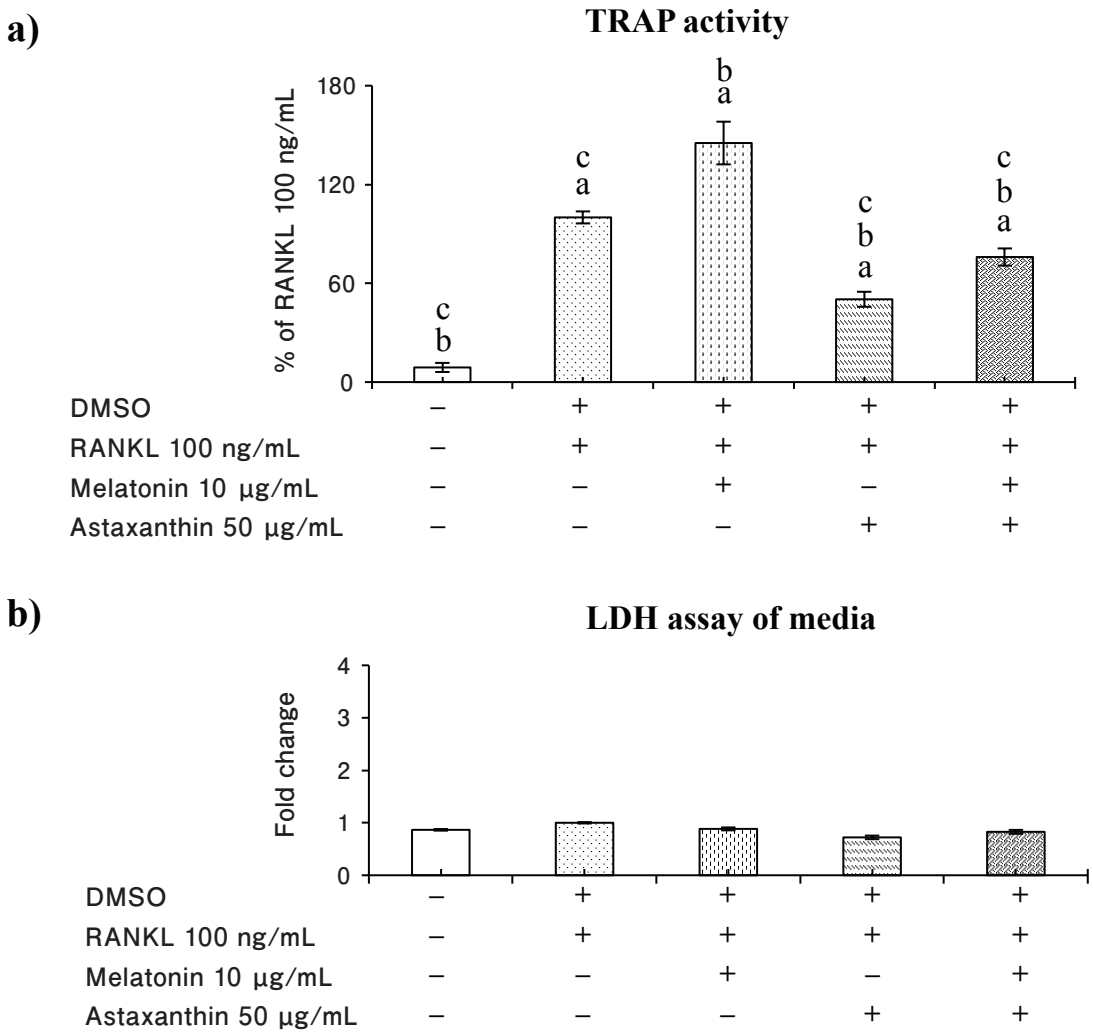

Fig. 4. **Effect of melatonin and astaxanthin together on TRAP activity.**

RAW264.7 cells were treated with RANKL 100 ng/mL along with melatonin in the presence and absence of astaxanthin for 5 days and then TRAP activity was measured. **a)** TRAP activity, **b)** LDH assay of media. All data are shown as means ± SEM, n = 6. a, b, c represent $p < 0.01$ vs αMEM, RANKL, RANKL + melatonin, respectively, Tukey-Kramer test. TRAP, tartrate resistant acid phosphatase; RANKL, receptor activator of nuclear factor kappa-B ligand; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; SEM, standard error mean.

Conclusion

Melatonin stimulated osteoclastogenesis and astaxanthin inhibited it (***Fig. 5***). Both showed potential to modulate the bone remodeling process. These findings may help to develop an effective drug or supplement against both bone formation and resorption mediated diseases.

Acknowledgments

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Conflict of interest

The authors claim no conflict of interest in this study.

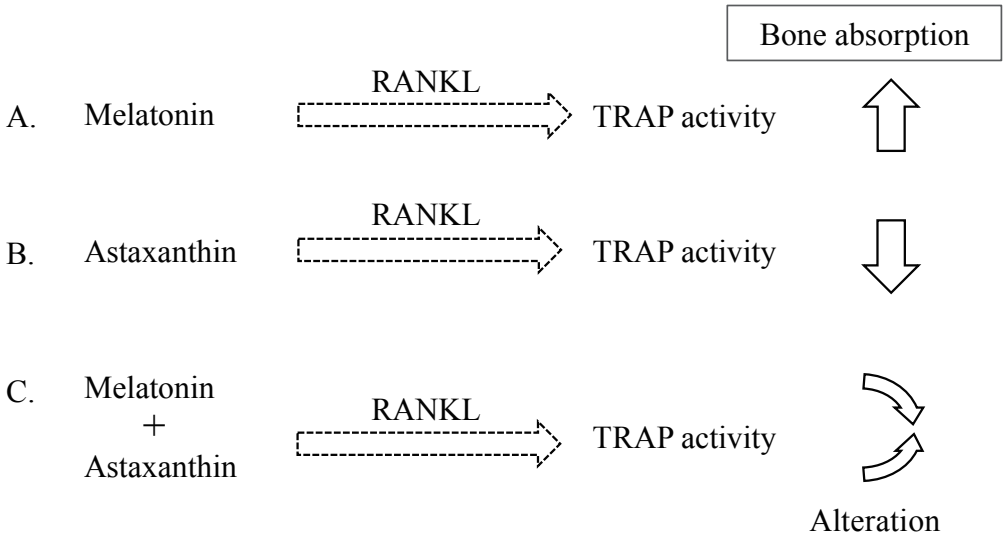

Fig. 5. **Summary.**

Schematic representation of "Effect of melatonin and astaxanthin on RANKL induced osteoclastogenesis in RAW264.7 cells".

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