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# The importance of estrogen for bone protection in experimental hyperthyroidism in human osteoblasts

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ABSTRACT

Triiodothyronine ($T_3$) and estrogen ($E_2$) play important roles in the bone remodeling process and signaling of receptor activator of the nuclear factor-kappa β (RANKL) and osteoprotegerin (OPG) expressed by osteoblasts. However, little is known of the molecular action of these hormones in conditions of hyperthyroidism and associated $E_2$ in human cells. AIMS: This study evaluated the effects of the physiological concentration of $E_2$ (10 nM), alone or in association with physiological (1 nM) and supraphysiological (10 nM) concentrations of $T_3$, on RANKL and OPG gene expression in human osteoblasts. MAIN METHODS: Alkaline phosphatase and osteocalcin assays were performed to verify the presence of mature osteoblasts. After mimicking the experimental hyperthyroidism in osteoblasts untreated or treated with $E_2$, RANKL and OPG gene expression was analyzed by real-time PCR and protein expression by western Blot and ELISA. Alizarin Red staining analyzed the amount of bone matrix after hormonal treatments. KEY FINDINGS: $E_2$ enhanced the gene expression of OPG when associated with 1 nM and 10 nM $T_3$. $E_2$ was able to restore the bone matrix after an initial decrease using 1 nM and 10 nM $T_3$. The protective effect of $E_2$ on the RANKL and OPG signaling pathway was demonstrated. E2 restored the bone matrix induced by experimental hyperthyroidism. SIGNIFICANCE: The data highlight the importance of $E_2$ to maintain OPG expression and osteoblast activity against possible loss of bone mass, especially in conditions where $T_3$ is in excess.

## 1. Introduction

Signaling of the receptor activator of the nuclear factor-kappa β (RANKL) and osteoprotegerin (OPG) expressed by osteoblasts is one of the most important pathways that act on bone remodeling [1,2]. Triiodothyronine ($T_3$) plays an important role in this process, regulating the differentiation and activity of osteoblasts and helping to maintain the integrity of the cell skeleton [3]. The action of $T_3$ at physiological concentration in vivo provides OPG levels sufficient to bind to RANKL by blocking the triggered resorption of bone [4]. However, the effect of this hormone on osteoblasts may be independent of this pathway [5,6].

Excess $T_3$, similar to hyperthyroidism, is an important factor that affects osteoblasts. In this condition, the bone remodeling cycle is imbalanced with a decrease in the formation of osteoblasts, leading to greater bone resorption and induction of osteoporosis. Although this response is established, the cellular and molecular mechanisms involved in this process are not clear [3].

Similar to $T_3$, estrogen ($E_2$) acts on osteoblasts by stimulating these cells to synthesize the bone matrix [7] and may promote increased OPG production by inhibiting bone resorption [8]. Some studies have verified the action of $T_3$ and $E_2$ by analyzing the potential for osteoblast differentiation, expression of genes involved in bone metabolism, and the capability of bone restoration in experiments with rats or cultures of MC3T3-E1 and osteoblasts or rat osteosarcoma (ROS 17/2.8) cells. [5,6,9,10]. Considering the importance of these hormones in bone metabolism, the lack of studies on human cell cultures is a critical gap in understanding the pathophysiological and molecular mechanisms related to hyperthyroidism and the possible restoration of bone loss through estrogen via RANKL and OPG.

From our study model of human osteoblasts [11], we evaluated the

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biological factors involved in this process. Our objective was to evaluate the effects of the $E_2$ physiological concentration associated with the different concentrations of $T_3$ (physiological and supraphysiological) on RANKL and OPG gene expression in human osteoblasts derived from mesenchymal stem cells. The findings indicate a protective effect of physiological $E_2$ in the reduction of bone mass induced by experimental hyperthyroidism via RANKL and OPG signaling.

## 2. Material and methods

### *2.1. Human osteoblast culture*

The Ethics Committee of Botucatu Medical School, São Paulo State University (UNESP) approved this study under number protocol 30458214.6.0000.541. All patients included in this study provided written informed consent. We used differentiated adipose stem cells (hASCs) obtained during abnominoplasty surgery from three donors. The hASCs were previously characterized [11]. The differentiation of hASCs in osteoblasts was performed using complete Dulbecco's Modified Eagle's Medium (DMEM) containing 0.1 μM of dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 50 μM of ascorbic acid (Gibco), and 10 mM of β-glycerophosphate (Sigma-Aldrich). The cells were maintained in this medium for 16 days as previously described [11]. The culture medium was replaced three times per week, maintaining the same osteogenic components. Undifferentiated hASCs were used as a control to confirm osteogenic induction.

### *2.2. Treatment with different $T_3$ doses and associations with $E_2$*

After the differentiation period, the osteoblasts were exposed to hormonal depletion for 12 h and maintained in DMEM supplemented with 10% charcoal-stripped fetal bovine serum (Sigma-Aldrich). The cells were then divided into groups for treatment with a physiological dose (1 nM) and a supraphysiological dose (10 nM) of $T_3$. Osteoblasts in the absence of $T_3$ were used as a control (C). Later, the previously isolated groups were associated with physiological doses of 10 nM $E_2$: 1 nM $T_3$ + 10 nM $E_2$ and 10 nM $T_3$ + 10 nM $E_2$. The treatments were performed for 72 h, with the treatment media replaced every 24 h.

### *2.3. Alkaline phosphatase and osteocalcin assay*

After 16 days of osteoinduction, 2 mL of the culture media were collected for alkaline phosphatase and osteocalcin assay using the immunoassay method according to the manufacturer's instructions (Cat. No. KAQ1381; Linco Research Inc., St. Charles, MO, USA). The culture medium of the undifferentiated hASCs was used as the negative control of osteoinduction.

### *2.4. Gene expression analysis*

Total RNA was extracted from the osteoblasts using the Trizol method (Invitrogen, São Paulo, Brazil). The High Capacity cDNA reverse transcription into RTPCR® kit (Invitrogen) was used to synthesize 20 μL of complementary DNA (cDNA) beginning with 1000 ng of total RNA. The mRNA levels of *RANKL* (Hs00243522_m1) and *OPG* (Hs00534382_m1) were determined by RT-qPCR. The quantitative measurements were performed in the Step One Plus detection system using the TaqMan commercial kit for qPCR (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The amplification conditions were as follows: enzyme activation at 50 °C for 2 min, denaturing at 95 °C for 15 min, and extension at 60 °C for 1 min. All the analyses were performed twice. The gene expression was quantified relative to the values of the control group, after normalization by an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm00434759_m1), by the 2-ΔΔCt method [12].

### *2.5. Western blotting*

The protein synthesis of RANKL and OPG was analyzed by western blotting. The proteins were lysed and homogenized with RIPA buffer (150 mM sodium chloride, 1% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 50 mM Tris, pH 8.0). The homogenate was centrifuged at 4 °C for 20 min at 15,300 × *g*. Quantification was performed according to the Bradford method following the standardized protocol in our laboratory. We used samples containing 25 ng of protein. Proteins were resolved by 15% SDS-polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane in a Mini-Trans Blot system using transfer buffer. Non-specific binding sites of the primary antibody to the membrane were blocked by incubation with blocking solution at room temperature with constant stirring. The membrane was then incubated overnight with the primary antibody at the following concentrations: anti-RANKL (Abcam, Cambridge, UK) 1:5000 and OPG 1:1000 (Abcam), with constant stirring at 2 to 8 °C. After incubation with the primary antibody, the membrane was incubated with the secondary antibody conjugated to peroxidase (anti-mouse or anti-rabbit IgG) diluted 1:1000 and 1:10000, respectively. Finally, immuno-detection was performed by the chemiluminescence method according to the manufacturer's instructions (Enhancer Chemi-Luminescence; Amersham Biosciences, Franklin Lakes, NJ, USA). Quantitative analyses of the protein bands (blots) were performed by Carestream Molecular Imaging 5.0 software (Carestream Health, Rochester, NY, USA).

### *2.6. Measurement of RANKL and OPG*

A commercially available ELISA kit (R & D Systems, Minneapolis, MN, USA) was to determine the concentrations of RANKL and OPG in the cell culture supernatant. The measurements were conducted in triplicate. The absorbance values were read at 450 nm on an ELISA automatic reader (Biotek Instruments Inc., Winooski, VT, USA). The standard curve was obtained in parallel to each assay and the results were converted in pg/mL. The intra- and inter-assay variability remained < 10.0% for RANKL and OPG.

### *2.7. Analysis of the bone matrix in the different $T_3$ concentrations and association with $E_2$*

After 16 days of osteogenic induction and treatment with the different $T_3$ concentrations, the matrix was stained with Alizarin S Red (Sigma-Aldrich). Cells were washed with phosphate buffered saline and fixed with 4% paraformaldehyde for 50 min at the room temperature at approximately 22 °C to 27 °C. Then the cells were incubated with Alizarin Red for 5 min. Alizarin Red was released from the cell matrix after incubation in 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 15 min. The Alizarin Red concentration was determined by measuring the absorbance at 562 nm.

### *2.8. Statistical analyses*

The data were assessed by the D'Agostino & Pearson omnibus normality test. For the alkaline phosphatase variables, the data were subjected to the Student's *t*-test. For the gene expression variables and protein synthesis, the data were subjected to ANOVA analysis of variance with Tukey's post hoc test. The significance level adopted was 5%, and the software used was the GraphPad Prism version 5.0 (GraphPad, La Jolla, CA, USA).

## 3. Results

### *3.1. Presence of alkaline phosphatase and osteocalcin in osteoblasts*

The alkaline phosphatase levels (Fig. 1A) and osteocalcin (Fig. 1B)

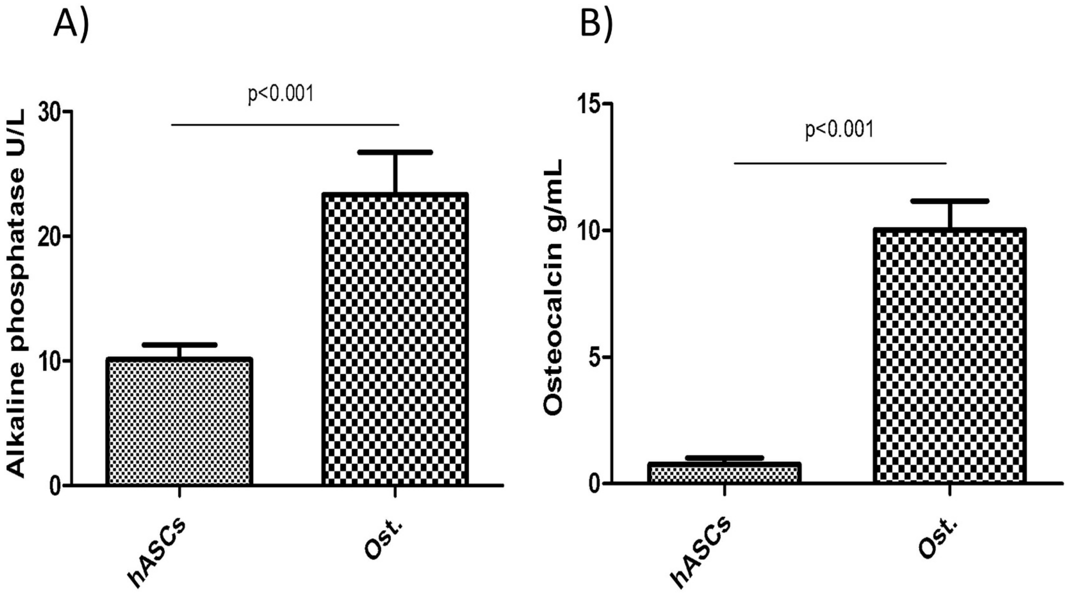

**Fig. 1.** Alkaline phosphatase (A) and osteocalcin (B) levels in osteoblasts after 16 days of osteoinduction. Human adipose tissue-derived stem cells are denoted as hASC and osteoblasts cultured for 16 days with osteoinduction medium as Ost. Osteoblasts and hASCs were cultivated for the same period. The data are expressed as mean ± standard deviation. *t*-test was used ($p < 0.001$); $n = 3$ for each treatment.

were elevated in the osteoblasts as compared with the undifferentiated hASCs, indicating cellular differentiation.

### 3.2. $T_3$ *increases mRNA and protein synthesis of RANKL and the physiological concentration of 1 nM* $T_3$ *increases mRNA and protein synthesis of OPG*

The 1 nM and 10 nM $T_3$ groups displayed elevated mRNA and protein levels of RANKL compared to the non-$T_3$ treated osteoblasts (Fig. 2A and B). We also noted an increase the mRNA of the 10 nM $T_3$ group compared to the 1 nM $T_3$ group (Fig. 2A). Increase mRNA and protein synthesis of OPG was evident at 1 nM $T_3$ in comparison with untreated $T_3$ osteoblasts (Fig. 2C and D). We also noted an increase the mRNA and protein synthesis of OPG in the 1 nM $T_3$ group compared to the 10 nM $T_3$ group (Fig. 2C and D).

### 3.3. $E_2$ *associated with 10 nM* $T_3$ *decreases RANKL mRNA and increases OPG mRNA when associated with 1 nM and 10 nM* $T_3$

The 1 nM and 10 nM $T_3$ groups were used as a control to assess the association with 10 nM $E_2$. $E_2$ at 10 nM potentiated the action of $T_3$ (1 nM), increasing the mRNA levels of RANKL (Fig. 3A). There was no difference in protein synthesis (Fig. 3B). $E_2$ at 10 nM decreased the expression of RANKL when associated with 10 nM $T_3$ (Fig. 3A). $E_2$ associated with 1 nM $T_3$ increased the level of mRNA and protein synthesis of OPG. The $E_2$ associated with 1 nM $T_3$ increased the mRNA level and protein synthesis of OPG, and when associated with 10 nM $T_3$ increased OPG mRNA levels, with no difference in protein synthesis (Fig. C and D).

### 3.4. $E_2$ *increases the amount of bone matrix that was decreased by the different concentrations of* $T_3$

The effect of different concentrations of $T_3$ in association with $E_2$ was investigated to verify the amount of bone matrix in the osteoblasts. The 10 nM concentration of $E_2$ increased the bone matrix compared to untreated osteoblasts (Fig. 4C). However, both 1 nM and 10 nM $T_3$ decreased the amount of the matrix in comparison with untreated $T_3$ osteoblasts (Fig. 4D and E). $E_2$ at 10 nM restored the amount of bone matrix that had previously been decreased with 1 nM or 10 nM $T_3$ (Fig. 4F and G).

## 4. Discussion

The effects of thyroid hormone on osteoblastic proliferation have been reported but remain controversial [13]. We previously described [11] the high mitochondrial membrane potential and absence of apoptotic and tumor cell processes in osteoblasts after 16 days culture in an osteoinduction medium. During the same time period, RANKL levels secreted by osteoblasts were increased, and we were able to demonstrate the maturation and development of these cells from hASCs [11]. This cytokine actively participates in the process of bone remodeling by binding to its OPG receptor on osteoblasts. The RANKL/OPG binding prevents exaggerated bone resorption while maintaining the integrity of the skeleton [14]. This interaction is the main signaling pathway produced by osteoblasts and has been investigated as a means of treating bone diseases [15]. In addition, systemic factors, such as triiodothyronine and $E_2$ regulate this signaling pathway to maintain normal bone remodeling. Presently, during the time of established osteoinduction and the presence of functional osteoblasts [11], we assessed the gene expression of RANKL and OPG in the presence of $T_3$ and $E_2$. The findings demonstrate importance in the RANKL and OPG bone remodeling process under normal or excessive hormonal conditions.

Initially, we found that the cells synthesized large quantities of alkaline phosphatase and osteocalcin, indicating that they had differentiated into osteoblasts (Fig. 1A and B, respectively), as has been previously described [11]. Therefore, they were able to deposit the mineralized matrix.

We observed increased gene expression of RANKL upon treatment with 1 nM and 10 nM $T_3$ compared to untreated osteoblasts (Fig. 2A and B). Our results agree with those reported by Miura et al. [9], who observed that $T_3$ at a supraphysiological concentration (10 nM) induced the expression of RANKL mRNA in primary culture of rat osteoblastic cells.

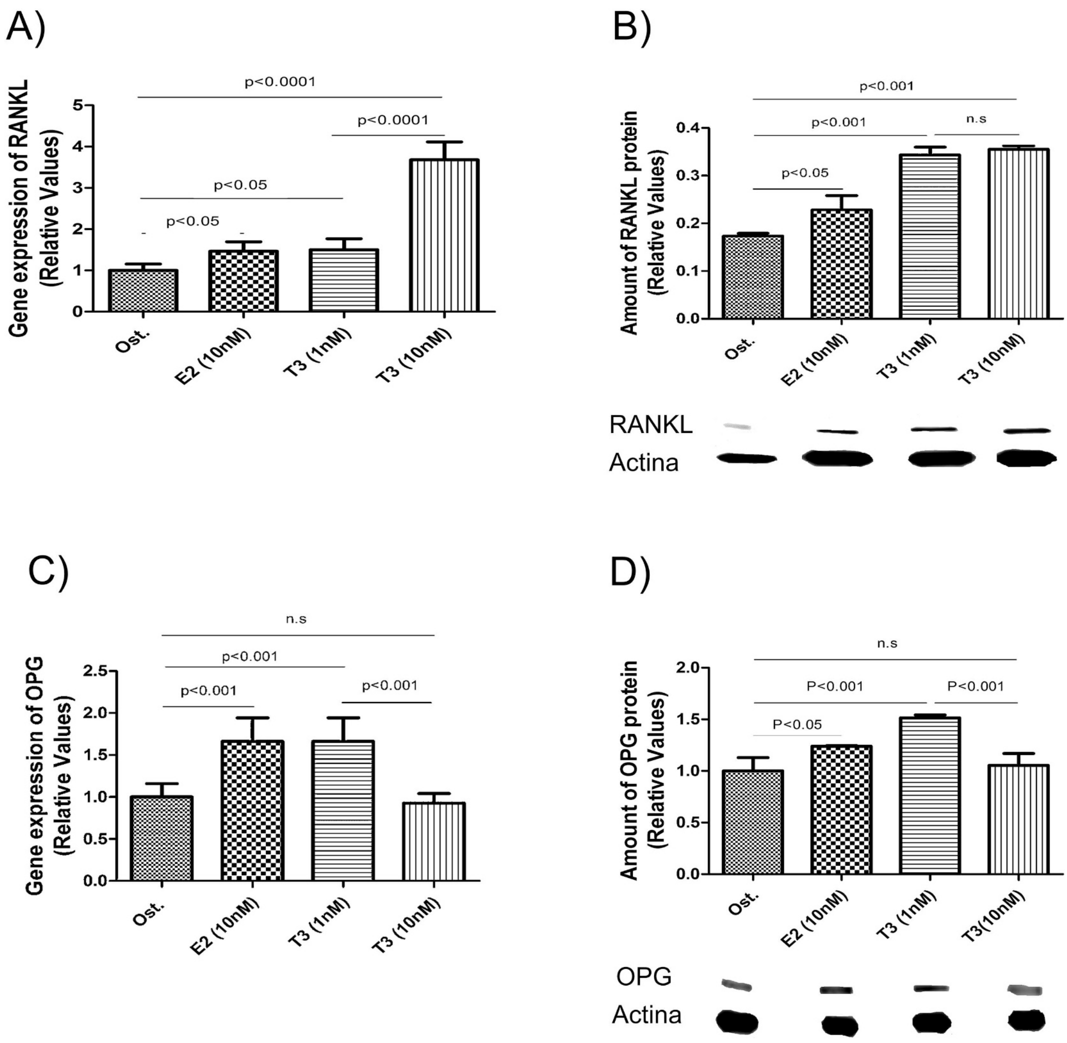

**Fig. 2.** Effect of the physiological dose of $E_2$ and different $T_3$ doses on the gene expression and protein synthesis of RANKL and OPG. (A) Gene expression of RANKL. (B) Amount of RANKL protein. (C) Gene expression of OPG. (D) Amount of OPG protein. (Ost) Osteoblasts cultured for 16 days with osteoinduction medium. ($E_2$ 10 nM) Osteoblasts with physiological dose of E2. (T3 1 nM) Osteoblasts with physiological dose of T3 (T3 10 nM) Osteoblasts with supraphysiological dose of T3. Hormone treatments were administered to osteoblasts for 72 h. n.s.: non-significant. Data are expressed as mean ± standard deviation (ANOVA) with Tukey's post hoc test, $P < 0.05$ was considered significant; $n = 3$ for each treatment.

The physiological dose of $T_3$ increased the mRNA as well as protein levels of OPG (Fig. 2C and D). Kanatani et al. [6] reported that when $T_3$ was administered in unfractionated bone cells rat, there was an increase in OPG mRNA levels and a decrease in RANKL mRNA levels in a dose-dependent manner. We verified that in the 1 nM $T_3$ group there was an increase levels of mRNA and protein synthesis of OPG in comparison with the 10 nM $T_3$ (Fig. 2C and D). OPG exerts its protective effect on bone resorption, acting as an antagonistic receptor to the effects of RANKL, especially in physiological situations. However, when a $T_3$ dose of 10 nM was administered, the levels of OPG mRNA and protein decreased and became similar to untreated osteoblasts (Fig. 2C and D). Thus, in the presence of the supraphysiological dose of $T_3$, osteoblasts can reduce the production of OPG, providing greater chances for RANKL binding to its receptor, which can activate osteoclastogenesis [16]. This data shows the importance of the protective effect of OPG on bone tissue in normal situations.

To understand the action of $E_2$ on osteoblasts in conjugation with the $T_3$ treatment, we explored the effect of an $E_2$ physiological dose with simulated euthyroidism and hyperthyroidism. We observed that the $T_3$ and $E_2$ associated treatments could modulate gene expression of RANKL and OPG. We demonstrated that 10 nM $E_2$ was adequate to increase thyroid hormone action. The 10 nM $T_3$ treatment associated with 10 nM $E_2$ was able to promote a significant reduction in RANKL mRNA levels and an increase in the OPG levels (Fig. 3A and C). There was an increase in the protein levels of OPG at the 1 nM concentration of $T_3$ with $E_2$. The finding confirms the restricted RANKL-OPG interaction in the control of bone remodeling [17]. The increase in OPG gene expression was evident in osteoblasts, especially in the different concentrations of $T_3$ associated with $E_2$. These results show that in such situations, osteoblasts may enhance OPG synthesis in an attempt to inhibit the action of RANKL. In a hyperthyroidism setting, the presence of $E_2$ is crucial for bone protection. Some authors explained that in this situation OPG appears to act by inducing osteoclastic apoptosis by binding to RANKL and preventing its binding to the receptor activator of nuclear factor-kappa B (RANK), restricting bone resorption [18,19].

In relation to bone matrix, $T_3$ can reduce the synthesis of non-collagen proteins, alkaline phosphatase, and osteocalcin, which are important in the process of matrix mineralization because they are produced by mature osteoblasts [20]. In this study, we noticed that this matrix was reduced when 1 nM and 10 nM $T_3$ were administered

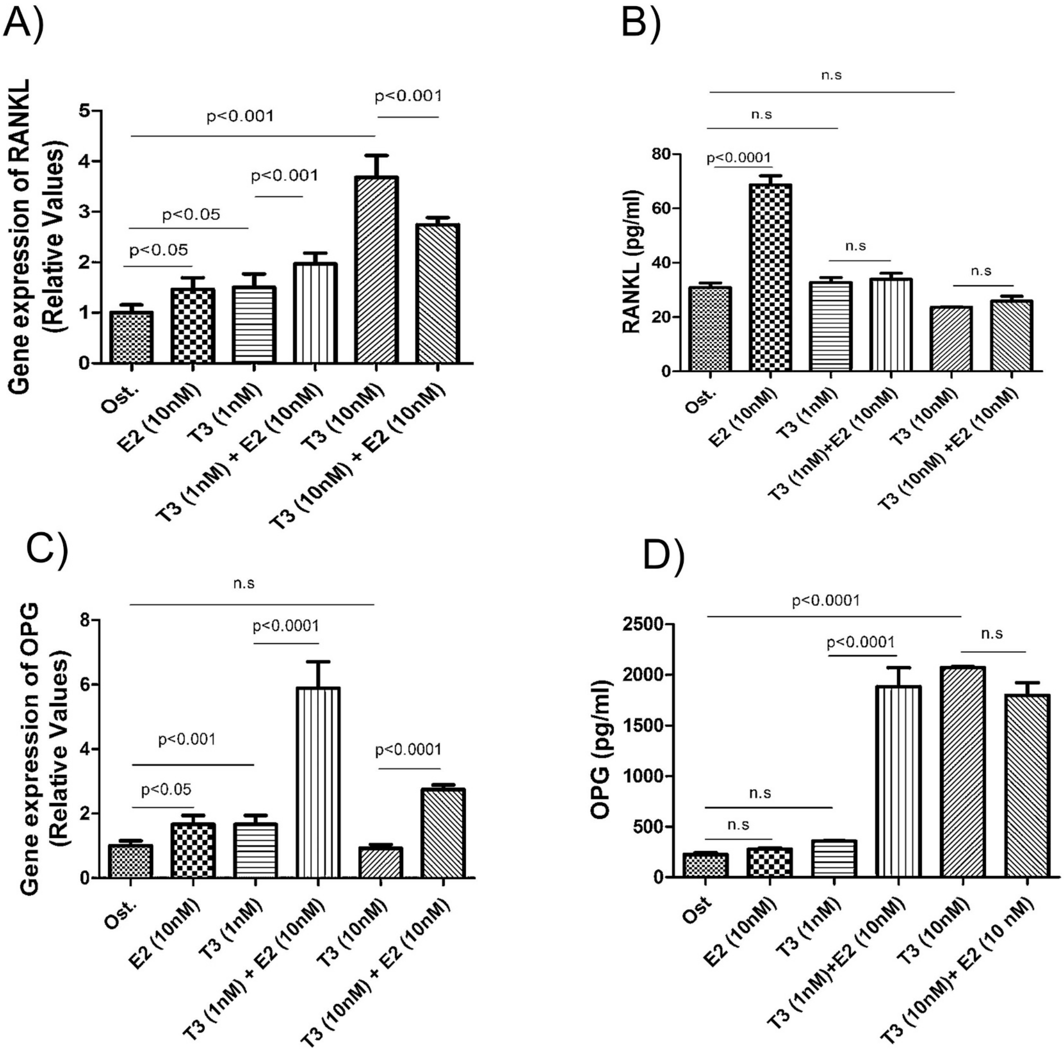

**Fig. 3.** Effect of the physiological and supraphysiological dose of T3 associated with the physiological dose of E2 on the gene and protein expression of RANKL and OPG. (A) Gene expression of RANKL. (B) Amount of RANKL protein. (C) Gene expression of OPG. (D) Amount of OPG protein. (Ost) Osteoblasts cultured for 16 days with osteoinduction medium. (E2) Osteoblasts with physiological dose of E2 (10 nM). (T3 1 nM) Osteoblasts with physiological dose of T3. (T3 10 nM) Osteoblasts with supraphysiological dose of T3. (T3 1 nM + E2 10 nM) Osteoblasts cultured in association with the physiological dose of T3 and E2. (T3 10 nM + E2 10 nM) Osteoblasts cultured in association with the supraphysiological dose of T3 and physiological E2. Hormone treatments were administered to osteoblasts for 72 h. Data are expressed as mean ± standard deviation (ANOVA) with Tukey's post hoc test, $P < 0.05$ was considered significant; $n = 3$ for each treatment.

(Fig. 4D, E and H), demonstrating that $T_3$ at these concentrations has a negative effect on the bone matrix. This supraphysiological concentration of $T_3$ may be similar to that occurring in vivo during hyperthyroidism, which reduces bone mass by induction of resorption and which may induce osteoporosis [21]. In addition to the supraphysiological dose, the physiological concentration of 1 nM may decrease or inhibit the synthesis of alkaline phosphatase, promoting decreased bone matrix and reduced osteoblast proliferation. These data were observed by Ernst and Froesch [22] when rat cells were treated with 1 and 10 nM $T_3$ for up to 20 days. This indicates the participation of $T_3$ in the production of this protein, which has an inhibitory effect on the bone matrix. However, the negative effect of physiological concentrations of thyroid hormone may be offset by the action of other hormones, such as $E_2$ and thyroid stimulating hormone. $E_2$ can increase the differentiation of osteoblasts and thereby stimulate bone matrix synthesis [23,24].

When 10 nM $E_2$ was used along with 1 nM and 10 nM of $T_3$, we noticed that $E_2$ restored the bone matrix in the presence of either concentration of $T_3$ (Fig. 4F, G and H). $E_2$ can regulate the synthesis of alkaline phosphatase, type I collagen, and osteocalcin in osteoblasts, thereby stimulating mineralized matrix formation [25].

In conclusion, the data demonstrate the protective effect of $E_2$ on the bone remodeling pathway of osteoblasts, as evident by the increase in OPG mRNA levels associated with 1 nM and 10 nM $T_3$. The findings provide important information regarding the proteins involved in bone remodeling in osteoblasts in the presence of $T_3$ with or without $E_2$ treatment. The 1 nM and 10 nM $T_3$ concentrations decreased the amount of mineralized bone matrix, but this matrix was restored in the presence of $E_2$. The association of $T_3$ and $E_2$ potentiated the expression of RANKL and OPG, except using the supraphysiological dose of $T_3$. The findings provide insights on the molecular action of $T_3$ as well as the interaction of $T_3$ and $E_2$ in the osteoblasts that are fundamental for bone remodeling. This knowledge may contribute to the understanding of the mechanisms involved in the reduction of bone mass observed in hyperthyroidism.

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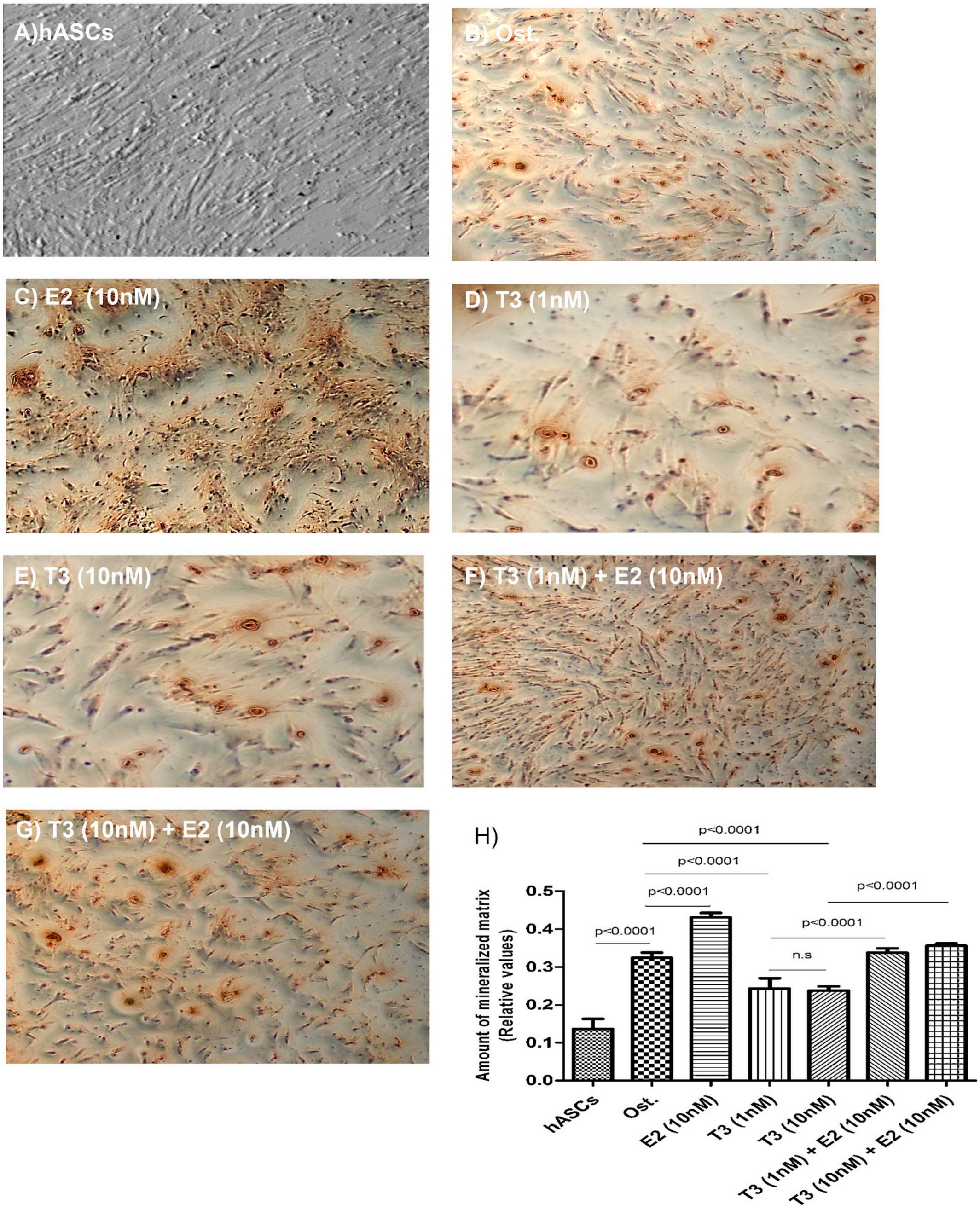

**Fig. 4.** Restoration of bone matrix in the presence of $E_2$. (A) hASCs without mineralized matrix. (B) Osteoblast bone matrix in the absence of $T_3$. (C) Bone matrix in the presence of 10 nM $E_2$. (D) Bone matrix in the presence of 1 nM $T_3$. (E) Bone matrix in the presence of 10 nM $T_3$. (F) Bone matrix in the presence of $T_3$ (1 nM + $E_2$ 10 nM). (G) Bone matrix in the presence of $T_3$ (10 nM + 10 nM $E_2$). (H) Graph of the amount of bone matrix in the different concentrations of $T_3$ in association with $E_2$. Human adipose tissue-derived stem cells are denoted as hASC. Osteoblasts cultured for 16 days with osteoinduction medium are denoted as Ost. Hormone treatments were administered to osteoblasts for 72 h. n.s: non-significant. Data are expressed as mean ± standard deviation (ANOVA) with Tukey's post hoc test. $P < 0.05$ was considered significant; $n = 3$ for each treatment.

## Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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