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Original Full Paper

In vitro effects of triiodothyronine on the reduced osteogenic potential of bone marrow mesenchymal stem cells of rats with osteoporosis

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Abstract

Objective: Evaluate the effect of *in vitro* triiodothyronine (T3) on the reduced osteogenic potential of bone marrow mesenchymal stem cells (BMMSCs) of adult rats with osteoporosis compared with BMMSCs of young and adult rats without osteoporosis. Methods: groups were tested: BMMSCs of young rats; BMMSCs of adult rats without osteoporosis; BMMSCs of adult rats with osteoporosis without T3 and BMMSCs of adult rats with osteoporosis treated with T3 (0.01, 1, 100 and 1000 nM). Alkaline phosphatase activity, MTT reduction, mineralized nodules and gene expression for collagen, osteocalcin, sialoprotein, osteopontin and BMP-2 were evaluated. Results: Osteoporosis increased the alkaline phosphatase activity and reduced the formation of mineralized nodules and expression of collagen and osteopontin in at least one of the observed time points. However, the T3 treatment of BMMSCs of rats with osteoporosis altered these parameters. Conclusion: It was concluded that doses of T3, 0.01 and 1000 nM had a positive effect promoted by increased osteogenic matrix synthesis and collagen expression in at least one of the evaluated time points compared to BMMSCs of rats with osteoporosis without treatment. However, T3 was unable to reach the osteogenic potential of the MSCs of healthy young rats.

Key words: stem cells, bone marrow, osteogenic differentiation, thyroid hormone, ovariectomy, rats.

Introduction

The effects of thyroid hormones are mainly due to the binding of T3 to the receptors TRα and TRβ, which are present on the cells of many tissues (16), including chondrocytes (4, 25), osteoblasts (1, 8) and osteoclasts (1). In culture of osteoblasts, the active hormone T3, in concentrations from 0.01 to 0.1 nM, is capable of stimulating proliferation and alkaline phosphatase activity, with the opposite effects found at higher concentrations (6). Furthermore, it was demonstrated that T3 also significantly stimulates osteogenic differentiation *in vitro* of bone marrow mesenchymal stem cells (BMMSCs) of healthy young rats in a dose-dependent manner (3, 9).

Adult stem cells, compared with embryonic stem cells, have a lower capacity for differentiation (5). The main advantage of the use of adult stem cells rests on the fact that the individual's own cells may be expanded in culture and again introduced into the patient without the risk of rejection by the immune system (24). Additionally, the adult stem cells present high capacity of cell proliferation in culture; ease of manipulation to replace

existing nonfunctional genes via gene splicing methods; ability to migrate to host target tissues (homing); and ability to integrate into host tissues and interact with the surrounding tissues (17). Consequently, the possibility of the use of adult stem cells for cell therapy has become an area of extensive research (14).

Osteoporosis is a disease characterized by low bone matrix synthesis caused by a failure of osteoblast function. Thus, any factor that reduces the synthesis of osteoblast activity can cause osteoporosis. Thus, sex hormone deficiency, thyroid and growth hormone deficiency, protein deficiency and loss of locomotor movements can result in osteoporosis. However, because the osteoblast cell originates from the differentiation of bone marrow mesenchymal stem cells, factors that reduce the osteogenic differentiation of the mesenchymal cells can also cause osteoporosis. BMMSCs of patients with osteoporosis have a deficiency in the production of collagen I (26). Additionally, Ocarino et al. (19) found that ovariectomized (OVX`d) rats deprived of sex steroids have reduced osteogenic potential of their BMMSCs, characterized by reduced synthesis of mineralized nodules. Furthermore, it was confirmed that injection of stem cells from the bone marrow of healthy isogenic rats into the femurs of rats with osteoporosis reverses local osteoporosis (20). It is known that injection of stem cells with the patient's own cells might have a better effect because there is no risk of rejection by the immune system. However, as the patient with osteoporosis displays a significant reduction in the osteogenic potential of their BMMSCs, it is postulated that the addition of T3 could increase the *in vitro* osteogenic differentiation of BMMSCs prior to the use of the cells in the treatment of osteoporosis. Therefore, the aim of this study was to evaluate the effect of *in vitro* treatment with T3 on the reduced osteogenic potential of mesenchymal stem cells of adult female rats with osteoporosis compared with the osteogenic potential of BMMSCs of young and adult rats without osteoporosis.

Materials and Methods

Ovariectomy and induction of osteoporosis

Twelve two-month-old female Wistar rats weighing 236.58 ± 20.42 g (mean ± SD) were used in this study. Animals from the same experimental group were housed together, with six rats per cage under a 12-h light/dark cycle. The rats were fed with commercial rat chow containing 22% crude protein, 1.4% calcium and 0.6% phosphorus. Food and water were provided *ad libitum* to all animals. The animals were initially randomly divided into two groups, one ovariectomized (OVX'd) (n=6) and the other sham-operated (control) (n=6). Bilateral ovariectomies were performed. This study was approved by the Ethical Committee of Universidade Federal de Minas Gerais.

Three months after the ovariectomy, a time period sufficient for the induction of osteoporosis (29) and confirmation by histomorphometric analysis, three ovariectomized rats and three control rats were euthanized. Trabecular bone percentage was determined by morphometry in longitudinal histological sections of long bones (proximal epiphysis and metaphysis of tibia, and distal epiphysis and metaphysis of femur). These variables were determined by using an ocular containing a grid with 121 points (Zeiss KPL 10×) and 20x magnification. The grid was superimposed across five fields in epiphysis and 10 fields in metaphysis. Fields were chosen at 1mm under the epiphyseal plate and articular cartilage. Then five-month-old rats were euthanized with an overdose of anesthetic (2.5% Tionembutal), and their right long bones (femur and tibia) were collected to determine the osteogenic potential of mesenchymal stem cells.

Cell harvesting and culture

Rat bone marrow mesenchymal stem cell cultures were established as previously described (3, 13, 18, 19, 30). In brief, the right femurs and tibias of adult rats (five-month-old) with and without osteoporosis and young rats (one-month-old) without osteoporosis (healthy) were dissected under aseptic conditions to remove the attached muscle and connective tissue. The epiphyses were removed and the bone marrow was flushed with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y., USA). The released cells were resuspended in DMEM supplemented with 10% fetal bovine serum (Gibco) plus antibiotics (60 µg/L gentamicin, 25 µg/L amphotericin B, 100 U/mL penicillin and 100 µg/mL streptomycin; Merck, Germany) and collected in a 75-cm^2 culture flask containing 10 ml of culture medium. The cells were grown at 37°C in 5% CO_2 for 3 days. The non-adherent cell population was removed, and the adherent layer was washed once with fresh medium. The culture medium was changed twice a week during culturing. Cells of the fourth passage were used for phenotypic characterization by FACS analysis.

BMMSCs of adult rats (five-month-old) with and without osteoporosis and of young rats (one-month-old) without osteoporosis were cultured in undifferentiated medium (DMEM) and osteogenic medium with and without 3,3',5-triiodo-L-thyronine (T3) depending on the group. BMMSCs cultured in osteogenic medium were separated into seven groups: 1) BMMSC of young rats without osteoporosis; 2) BMMSCs of adult rats without osteoporosis; 3) BMMSCs of adult rats with osteoporosis without T3; 4) BMMSCs of adult rats with osteoporosis with T3 (0.01 nM); 5) BMMSCs of adult rats with osteoporosis with T3 (1 nM); 6) BMMSCs of adult rats with osteoporosis with T3 (100 nM); and 7) BMMSCs of adult rats with osteoporosis with T3 (1000 nM). The following parameters were then evaluated: alkaline phosphatase activity, conversion of MTT into formazan

crystals, quantification of gene transcripts for collagen I, osteocalcin, bone sialoprotein, osteopontin and BMP-2 by real-time RT-PCR at 7, 14 and 21 days, and the percentage of mineralized nodules at 21 days of differentiation. All *in vitro* assays were conducted with six replicates per group and at each time point as described in detail below.

FACS analysis

Cells from the fourth passage from young and adult rats without osteoporosis and adult rats with osteoporosis were harvested with trypsin/EDTA (ethylene diamine tetra-acetic acid) and centrifuged at 1,400 x g for 10 min. The cells were then resuspended at a density of 1×10^6 cells/well in phosphate-buffered saline (PBS). The cell aliquots were incubated with individual primary or control antibodies for 30 min at 4°C. The cells were washed in PBS and incubated with a fluorophore-conjugated secondary antibody (Alexa Fluor 488, Molecular Probes, OR, USA) for 30 min at 4°C. The samples were analyzed using a FACScan cytometer (Becton Dickinson, NY, USA), and the data were analyzed using Cellquest software (Becton Dickinson). The following primary antibodies were used: anti-CD45 (clone 69 mouse), anti-CD54 (clone 1A29 mouse), anti-CD73 (clone 5 F/B9 mouse), and anti-CD90 (clone Ox-7 mouse) (BD Biosciences, San Jose, CA, USA).

Culture of osteoblasts

Calvaria osteoblast cultures were used as a positive control for differentiated MSC. Osteoblast cultures were established as previously described (31). In brief, the heads of three 2-day-old neonatal Wistar rats were separated from the body and then immersed in PBS. Calvaria were then dissected out and processed for cell isolation and culture. The central parts of the parietal and frontal bones, with their endosteum and periosteum, were incubated for 15 min with 1% trypsin and then for 60 min at 37ºC in PBS with 0.25% collagenase (Sigma, St Louis, MO; Type I). The cells dissociated from the bone fragments were washed several times in PBS, suspended in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) plus antibiotics (60 µg/L gentamicin, 25 µg/L amphotericin B, 100 U/mL penicillin and 100 µg/mL streptomycin; Merck, Germany) and collected in a 75-cm^2 culture flask containing 10 mL of culture medium. The cells were grown at 37°C and 5% CO_2 for 3 days. The non-adherent cell population was removed, and the adherent layer was washed once with fresh medium. The culture medium was changed twice a week during culturing (31). Cells from the fourth passage were used for RNA extraction to analyze the expression of collagen and non-collagen proteins by quantitative real-time PCR (qRT-PCR).

Viability assay

Before being cultured in osteogenic medium, the bone marrow mesenchymal stem cells of young and adult rats without osteoporosis and of adult rats with osteoporosis were tested for viability using the trypan blue stain. In brief, the cells were cultured in a 75-cm^2 culture flask ($1x10^4$ cells/cm^2) in DMEM supplemented with 10% fetal bovine serum plus antibiotics. During the test, the cells were harvested with trypsin/EDTA, centrifuged at 1,400 x g for 10 min, and stained with trypan blue. The nonviable (blue) and viable (transparent) cells were quantified in a Neubauer chamber.

Osteogenic differentiation

After the fourth passage, the adherent cells were harvested by treatment with trypsin/EDTA. The cells were then counted and plated in 6- and 24-well culture plates and in 25-cm^2 culture flasks (1×10^4 cells/cm^2). The cells of adult rats with and without osteoporosis and the cells of young rats without osteoporosis were cultured in osteogenic medium, which consisted of DMEM supplemented with 10% fetal bovine serum (Gibco), 60 µg/L gentamicin, 25 µg/L amphotericin B, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM β-glycerophosphate, 50 µg/mL ascorbic acid (Merck, Germany) and 10 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). The cells were grown at 37°C in 5% CO_2. The BMMSCs of adult female rats with osteoporosis were also cultured with different doses of 3,3',5-triiodo-L-thyronine (0.01, 1.0, 100 and 1000 nM; Sigma-Aldrich). Doses of 3,3',5-triiodo-L-thyronine were established according to a study conducted by Ishida et al. (11) and Boeloni et al. (3), with the dose of 0.01 nM similar to the physiological dose. The cells were grown for 7, 14 and 21 days to assess the conversion of MTT into formazan crystals, alkaline phosphatase activity, and gene expression of collagen I, osteocalcin, bone sialoprotein, osteopontin and BMP-2 via qRT-PCR and were grown for 21 days to assess the production of mineralized nodules.

MTT reduction assay

Briefly, the cells were incubated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for 2 h at 37°C. After incubation with MTT, the cells were treated for 12 h with a solubilization solution of sodium dodecyl sulfate (SDS) in 10% HCl, and the absorbance at 595 nm for the solubilized MTT formazan product was measured using a microtiter plate reader (31). The mean absorbance and standard deviation (SD) were determined from six replicates for each experimental group.

Alkaline phosphatase activity

Briefly, the cells were incubated with the BCIP/NBT solution (Zymed Laboratories, CA, USA) for 2 h at 37°C. After incubation, the cells were treated for 12 h with the solubilization solution (SDS in 10% HCl). The absorbance at 595 nm was measured using a microtiter plate reader (3, 19), and the mean absorbance and SD were determined from six replicates for each experimental group.

Mineralization assay

Briefly, the cells were washed in PBS, fixed in 70% ethanol and rinsed with deionized water. After the addition of a 5% silver nitrate solution, the wells were exposed to light for 2 h. The plates were rinsed with deionized water, and the residual silver nitrate was neutralized by 5% sodium thiosulfate, which stains calcium deposits dark brown. The cells were counterstained with eosin. The percentage of the mineralized nodules was assessed by light microscopy and was quantified in 50 fields using an ocular piece containing a 121-point grid (Zeiss KPL 10x) with a 20x magnification (31). The mean and SD were determined from six replicates for each experimental group.

quantitative RT-PCR (qRT-PCR)

The relative quantification of the gene expression of collagen I, osteocalcin, bone sialoprotein, osteopontin and BMP-2 in the BMMSC cultures was compared among the seven experimental groups. The groups were compared with calvaria osteoblast cultures as a positive control for differentiated cells. The calvaria osteoblasts was used as the calibrator. The mean and SD were determined in quadruplicate for each experimental group.

Total mRNA was extracted by adding the Trizol reagent (Gibco) according to the manufacturer's instructions. One microgram of RNA was subjected to cDNA synthesis using a SuperScript III Platinum Two-Step qPCR kit with SYBR Green (Invitrogen). The qRT-PCR reactions were performed in a Smart Cycler II thermocycler (Cepheid Inc.). The one-step qRT-PCR amplification started with reverse transcription for 120 sec at 50°C, followed by PCR with the following parameters: 45 cycles of 15 sec at 95°C and 30 sec at 60°C. At the end of each run, the fluorescence data were analyzed to obtain CT values. Gene expression was calculated using the 2-ΔΔCt method, where the values from the samples were averaged and calibrated with respect to the GAPDH CT values. The primers for the rat genes were as follows: sense 5'-GCAAGGTGTTGTGCGATGACG-3', antisense 5'-GGGAGACCACGAGGACCAGAG-3' for *collagen I*; sense 5'-CATCTATGGCACCACCGTTT-3', antisense 5'-AGAGAGAGGGAACAGGGAGG-3' for *osteocalcin*; sense 5'-TGTCCTTCTGAACGGGTTTC-3', antisense 5'-CTTCCCCATACTCAACCGTG-3' for *bone sialoprotein*; sense 5'-ATCTCACCATTCCGATGAATCT-3', antisense 5'-TCAGTCCATAAGCCAAGCTATCA-3' for *osteopontin*; sense 5'-TAGTGACTTTTGGCCACGACG-3', antisense 5'-GCTTCCGCTGTTTGTGTTTG-3' for *BMP-2*; and sense 5'-CAACTCCCTCAAGATTGTCAGCAA-3', antisense 5'-GGCATGGACTGTGGTCATGA-3' for *GAPDH*.

Statistical analysis

Delineation was entirely at random with a 7 x 3 factorial (seven groups x three time points). The data were submitted to analysis of variance, and the means were compared using the Student-Newman-Keuls Test (Instat, version 3.00, 32 Win 95/NT; GraphPad Software San Diego, CA, USA). Differences were considered significant if $p < 0.05$.

Results

Morphometric analysis

The OVX'd group showed lower trabecular bone percentage of proximal metaphysis of tibia (16.42 ± 10.30) compared to the sham-operated (control) group (37.17 ± 6.99). The OVX'd group showed lower trabecular bone percentage of distal metaphysis of femur (16.90 ± 1.63) compared to the sham-operated (control) group (45.39 ± 9.14).

Phenotypic characterization of bone marrow MSCs

The bone marrow mesenchymal stem cells of young and adult rats without osteoporosis and adult rats with osteoporosis had similar phenotypic characterization and showed expression of CD45 in a maximum of 3.06% of the cells and expression of CD54, CD73, intercellular adhesion molecule-1, (ICAM-1) and CD90 in a minimum of 84.27% of the cells (Table 1).

Table 1. Percentage expression of CD45, CD54, CD73, and CD90 by FACS analysis in bone marrow mesenchymal stem cells of young and adult female rats without osteoporosis and adult female rats with osteoporosis.

Groups	CD45	CD54	CD73	CD90
Young female rats without osteoporosis	3.06	95.10	93.99	86.77
Adult female rats without osteoporosis	1.09	96.94	84.27	95.93
Adult female rats with osteoporosis	1.01	97.94	92.20	95.99

Viability assay by trypan blue stain

After the phenotypic characterization and before the osteogenic differentiation, the cultures of MSCs of all groups demonstrated 100% viability.

MTT conversion into formazan crystals

At day 7 of differentiation, the BMMSCs of rats with osteoporosis without T3 treatment presented smaller capacities for the conversion of MTT into formazan crystals in comparison to the BMMSCs of rats without osteoporosis. Compared with cells from rats with osteoporosis, T3 treatment increased the conversion of MTT to formazan only at doses of 1 nM at 7 and 21 days to levels higher than those found in the cultures of BMMSCs of young rats at day 7 and to levels similar to those found in the cultures of BMMSCs of young rats at 21 days. The remaining doses of T3 (0.01, 100 and 1000 nM) reduced the activity of MTT only at day 7 (Fig. 1A-C). At day 21 of differentiation, the BMMSCs of young rats showed higher MTT conversion than the BMMSCs of rats without osteoporosis (Fig. 1C).

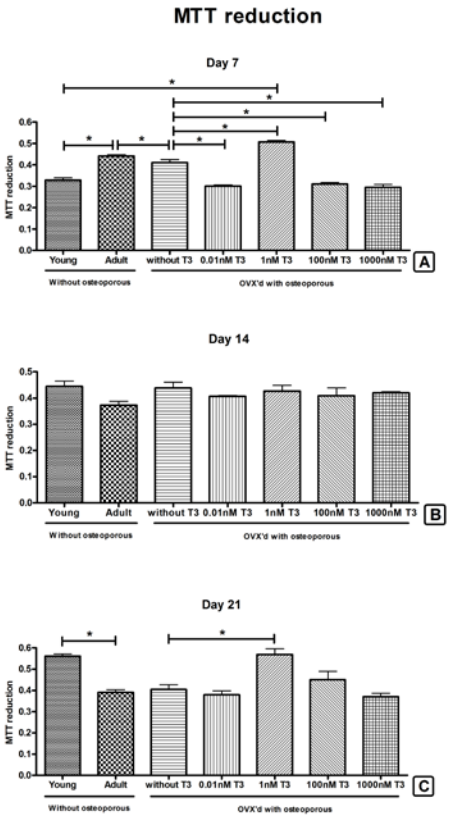

Figure 1. The MTT reduction into formazan crystals in the BMMSCs. MTT reduction into formazan crystals (mean ± SD absorbance at 595 nm) in BMMSCs from young and adult female rats without osteoporosis and adult female rats with osteoporosis with and without T3 treatment after 7 (A), 14 (B) and 21 (C) days of differentiation. * $p<0.05$.

Alkaline phosphatase activity

At all evaluated periods, the BMMSCs of young rats showed higher alkaline phosphatase activity than the BMMSCs of rats without osteoporosis. Only at day 7 of differentiation, the cells of adult rats with osteoporosis presented increased alkaline phosphatase activity compared to the BMMSCs of adult rats without osteoporosis. Treatment of the BMMSCs with T3 did not change the alkaline phosphatase activity at all time points, except at 7 and 14 days, in which the cells of adult rats with osteoporosis treated with 1 nM T3 showed smaller activity of this enzyme compared with the osteoporosis untreated group (Fig. 2A-C).

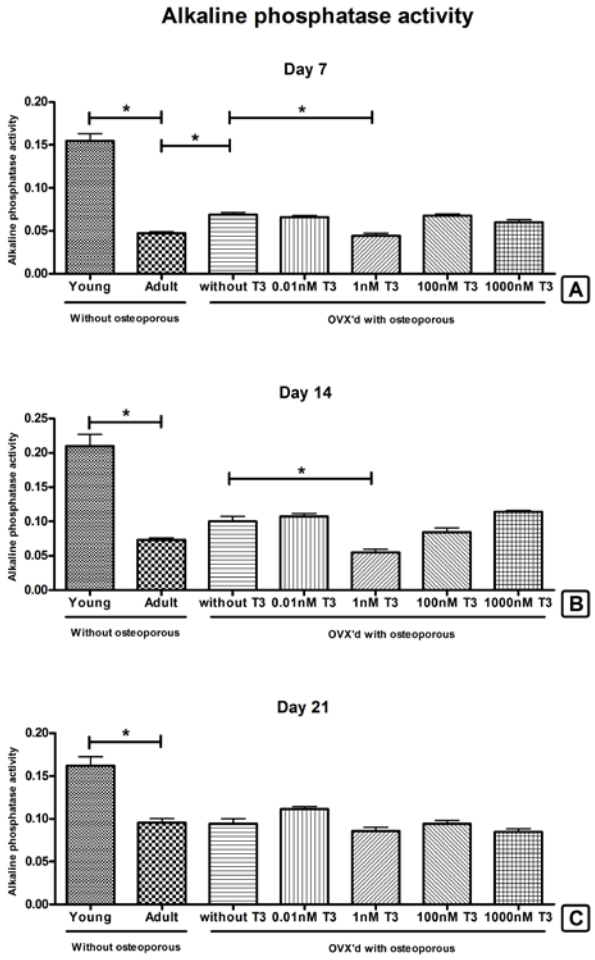

Figure 2. The alkaline phosphatase activity in the BMMSCs. Alkaline phosphatase activity (mean ± SD absorbance at 595 nm) in BMMSCs from young and adult female rats without osteoporosis and adult female rats with osteoporosis with and without T3 treatment after 7 (A), 14 (B) and 21 (C) days of differentiation. * $p<0.05$.

Percentage of mineralized nodules per field

The BMMSCs of rats without osteoporosis showed smaller percentage of mineralized nodules per field compared with the BMMSCs of young rats. The effect of osteoporosis was also observed on the synthesis of mineralized nodules, with a significant reduction compared to cultures of BMMSCs of adult rats without osteoporosis. However, the T3 treatment positively affected this result, significantly increasing the percentage of mineralized nodules per field at the doses of 0.01 and 1000 nM, reaching similar values to that of the BMMSCs cultures of adult rats without osteoporosis but not equaling the values of the cultures of the BMMSCs of young rats (Fig. 3 and 4).

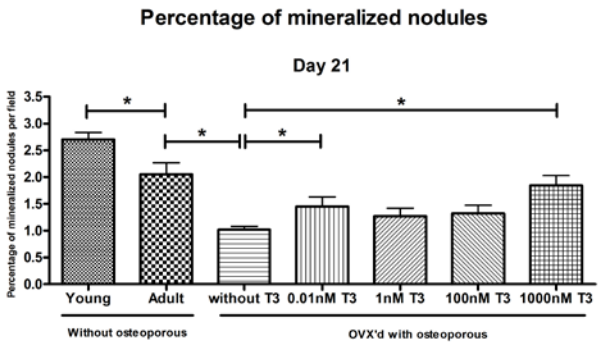

Figure 3. The percentage of mineralized nodules per field produced by BMMSCs. Percentage of mineralized nodules per field (mean ± SD absorbance at 595 nm) in BMMSCs from young and adult female rats without osteoporosis and adult female rats with osteoporosis with and without T3 treatment after 7 (A), 14 (B) and 21 (C) days of differentiation. * $p<0.05$.

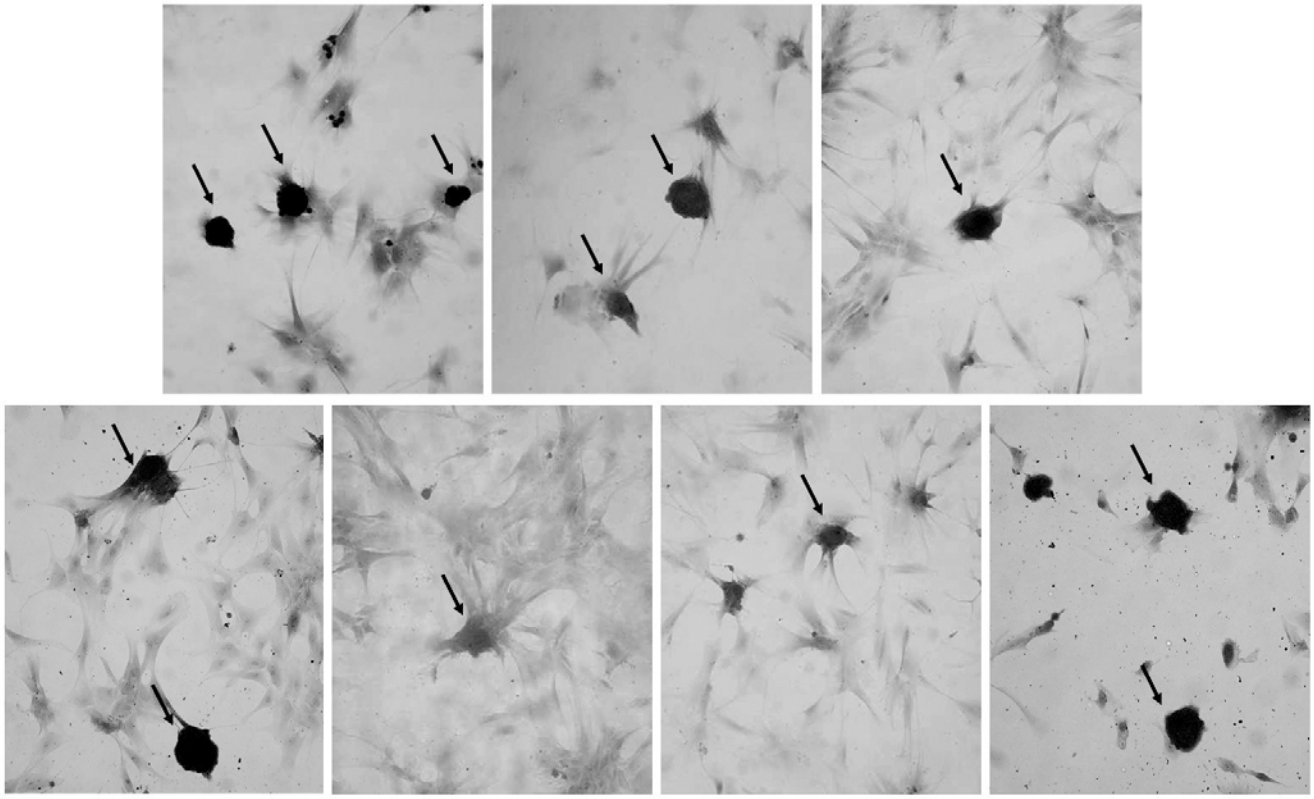

Figure 4. The mineralized nodules produced by BMMSCs. Mineralized nodules (arrows) in BMMSCs from young and adult female rats without osteoporosis and adult female rats with osteoporosis with and without T3 treatment after 21 days of differentiation. BMMSCs of young rats without osteoporosis (A), BMMSCs of adult rats without osteoporosis (B), BMMSCs of adult rats with osteoporosis without T3 (C), BMMSCs of adult rats with osteoporosis with T3 at 0.01 nM (D), 1 nM (E), 100 nM (F) and 1000 nM (G). Bar = 89.85 µm.

Quantitative RT-PCR (qRT-PCR)

The expression of collagen I at day 7 of differentiation was higher in the majority of the groups than in the osteoblasts, except in cells from the group with osteoporosis treated with 100 nM T3 (Fig. 5A). At 14 days, only the BMMSCs of young and adult rats with osteoporosis treated with 1000 nM T3 showed higher collagen I expression than the osteoblasts (Fig. 5B). However, at 21 days of differentiation, collagen I

expression was lower in the majority of groups when compared to the osteoblasts, except for the BMMSCs of young rats (Fig. 5C).

The effect of osteoporosis on the reduced collagen I expression was only observed after 14 days of differentiation (Fig. 5B). Interestingly, compared with the BMMSCs of rats with osteoporosis, hormonal treatment with T3 increased the collagen I expression at doses of 0.01, 1 and 1000 nM at 14 days of differentiation (Fig. 5B).

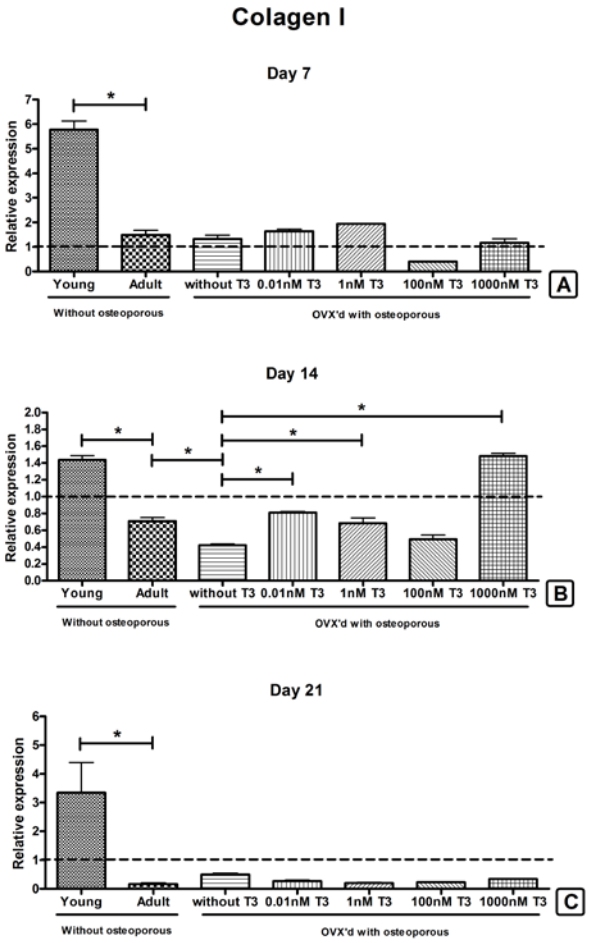

Figure 5. The relative quantification of collagen I gene expression via qRT-PCR. Relative quantification of collagen I gene expression (mean ± SD) via qRT-PCR in the BMMSCs from young and adult female rats without osteoporosis and adult female rats with osteoporosis with and without T3 treatment after 7 (A), 14 (B) and 21 (C) days of differentiation. The data are expressed in relation to osteoblast cultures (stippled line). * $p<0.05$.

The osteocalcin expression was lower at all evaluated periods of differentiation and in all groups when compared with osteoblasts (Fig. 6A-C). Moreover, osteoporosis and treatment with T3 did not alter the osteocalcin expression at the time points evaluated (Fig. 6A-C).

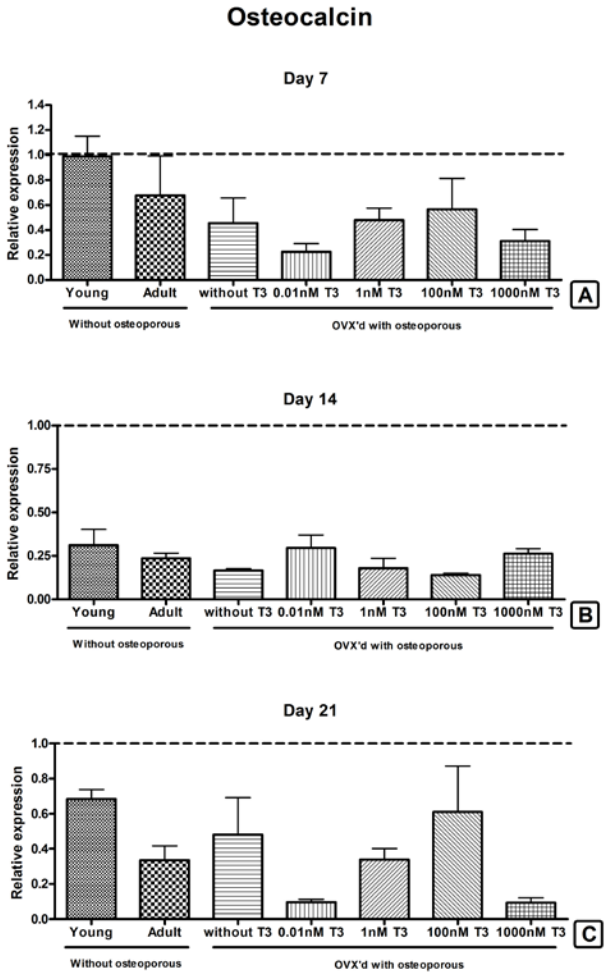

Figure 6. The relative quantification of osteocalcin gene expression via qRT-PCR. Relative quantification of osteocalcin gene expression (mean ± SD) via qRT-PCR in the BMMSCs from young and adult female rats without osteoporosis and adult female rats with osteoporosis with and without T3 treatment after 7 (A), 14 (B) and 21 (C) days of differentiation. The data are expressed in relation to osteoblast cultures (stippled line). * $p<0.05$.

The bone sialoprotein expression was higher when compared to the osteoblast cultures at all evaluated periods of differentiation in the BMMSCs of young rats (Fig. 7A-C). Furthermore, osteoporosis and treatment with T3 did not alter the bone sialoprotein expression at the time points evaluated (Fig. 7A-C).

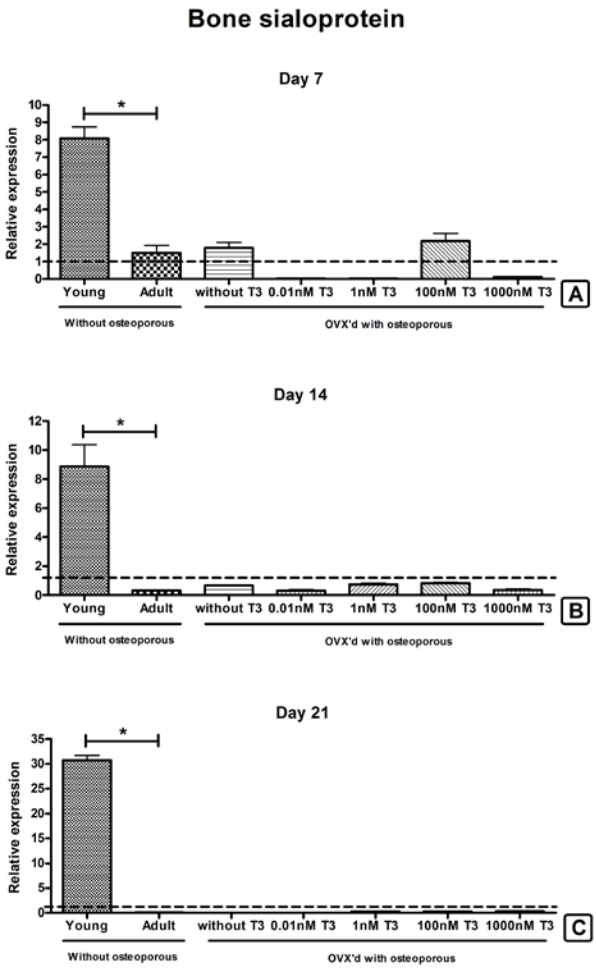

Figure 7. The relative quantification of bone sialoprotein gene expression via qRT-PCR. Relative quantification of bone sialoprotein gene expression (mean ± SD) via qRT-PCR in the BMMSCs from young and adult female rats without osteoporosis and adult female rats with osteoporosis with and without T3 treatment after 7 (A), 14 (B) and 21 (C) days of differentiation. The data are expressed in relation to osteoblast cultures (stippled line). * $p<0.05$.

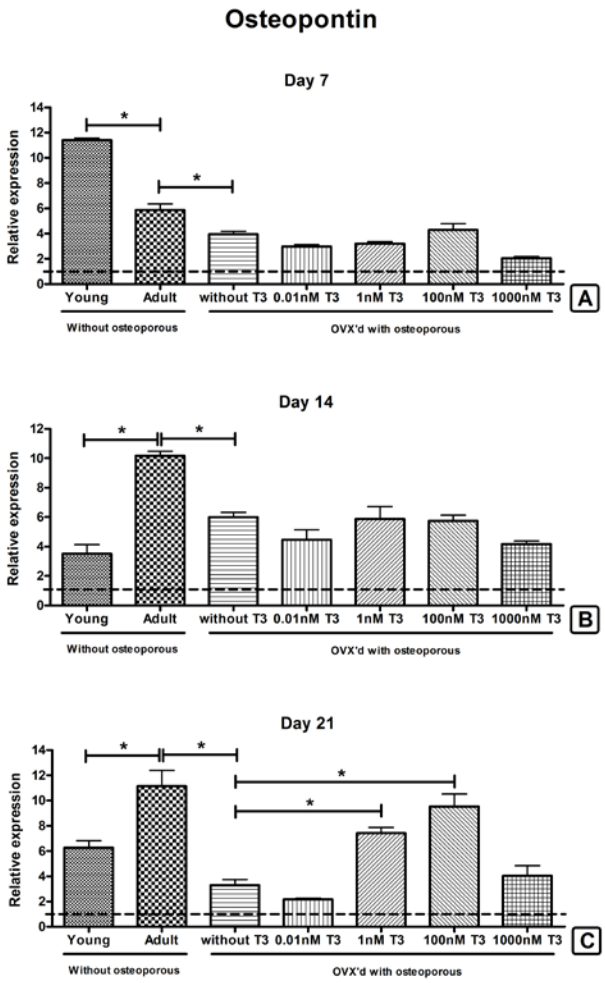

Figure 8. The relative quantification of osteopontin gene expression via qRT-PCR. Relative quantification of osteopontin gene expression (mean ± SD) via qRT-PCR in the BMMSCs from young and adult female rats without osteoporosis and adult female rats with osteoporosis with and without T3 treatment after 7 (A), 14 (B) and 21 (C) days of differentiation. The data are expressed in relation to osteoblast cultures (stippled line). * $p<0.05$.

Furthermore, the effect of osteoporosis on the osteopontin expression was observed at three differentiation time points; the BMMSCs of adult female rats with osteoporosis showed osteopontin expression significantly lower when compared with cells of adult female rats without osteoporosis at all evaluated periods (Fig. 8A-C). Interestingly, hormonal treatment with T3 only increased the osteopontin expression at the doses of 1 and 100 nM compared with the BMMSCs of rats with osteoporosis without T3 at 21 days of differentiation (Fig. 8C). The expression of osteopontin was higher at all evaluated periods of differentiation and in all groups when compared with osteoblasts (Fig. 8A-C).

The BMP-2 expression was lower when compared to the osteoblast cultures at 7 and 14 days of differentiation in all groups studied (Fig. 9A-B). At 21 days of differentiation, BMP-2 expression was lower in the majority of the groups when compared with osteoblasts, except in cells from the young group and the group with osteoporosis (Fig. 9C). Moreover, there was no effect of osteoporosis in the BMP-2 expression at the time points evaluated (Fig. 9A-C). However, compared with cells from rats with osteoporosis, the T3 treatment increased the BMP-2 expression at doses of 0.01 nM, 1 nM and 100 nM after 14 days of differentiation (Fig. 9A-C).

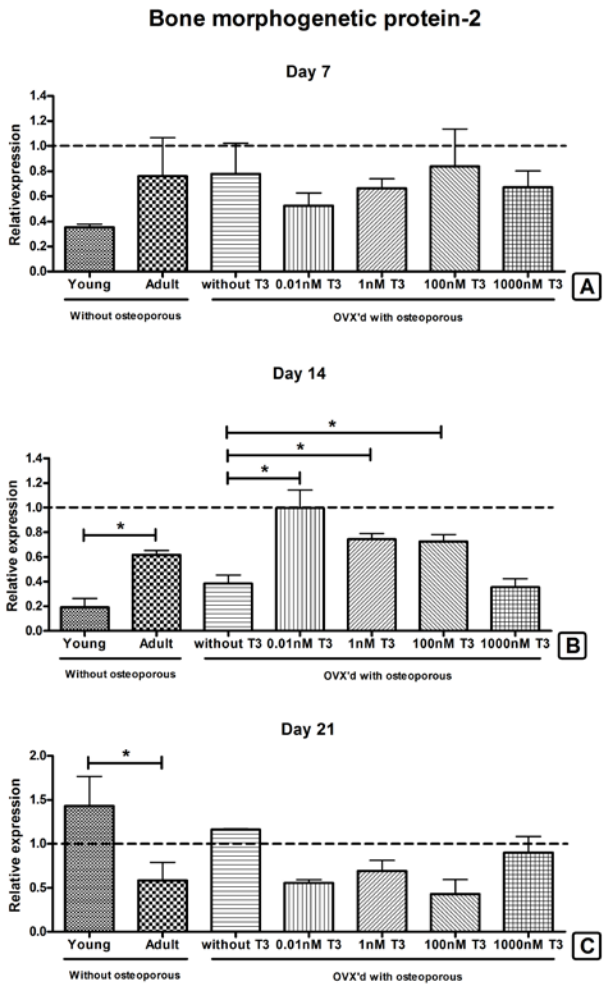

Figure 9. The relative quantification of bone morphogenetic protein-2 gene expression via qRT-PCR. Relative quantification of bone morphogenetic protein-2 gene expression (mean ± SD) via qRT-PCR in the BMMSCs from young and adult female rats without osteoporosis and adult female rats with osteoporosis with and without T3 treatment after 7 (A), 14 (B) and 21 (C) days of differentiation. The data are expressed in relation to osteoblast cultures (stippled line). * $p<0.05$.

Discussion

Regardless of the group, the MSCs had similar characteristics with respect to surface markers (i.e., expressed CD54, CD73 and CD90 and very little CD45). This characterization is important because the bone marrow contains MSCs, hematopoietic cells (2) and fibroblasts (12). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has proposed that MSCs must express CD73 and CD90 but not CD45 (28). Hematopoietic cells express CD45 (2), which can also be expressed in fibroblasts (27). CD54 (ICAM-1) is not exclusive to MSCs and is expressed at low concentrations in leukocytes and endothelial cells (27). Despite this, leukocytes and other hematopoietic cells do not adhere to plastic (21). Additionally, in the bone marrow, only MSCs demonstrate the capacity for osteogenic differentiation (2, 21).

As expected, osteoporosis has altered the osteogenic potential of BMMSCs. Interestingly, the results of treatment with T3, although varying with the dose and the time point, suggest an improvement of the osteogenic potential of BMMSCs of rats with osteoporosis. However, treatment with T3 was unable to reach the osteogenic potential of MSCs in rats with osteoporosis when compared with healthy young rats. The results in BMMSCs from adult without and with osteoporosis were not different if they were evaluated in rats with more than three months of ovariectomy, because it is a time sufficient for the induction of osteoporosis in rats in agreement with Serakides et al. (29), Ocarino et al. (19) and Yang et al. (34).

The BMMSCs of rats with osteoporosis demonstrated lower osteogenic differentiation, characterized by the reduction in the formation of mineralization nodules and in the expression of collagen I and osteopontin in at least one of the time points when compared with the BMMSCs of adult rats without osteoporosis. Ocarino et al. (19) also found that the BMMSCs of rats with osteoporosis have a lower capacity to synthesize mineralized matrix and even suggested that the reduction of the osteogenic differentiation of BMMSCs may be one of the mechanisms involved in the pathogenesis of the osteoporosis resulting from a sex steroid deficiency. The importance of sex hormones in the osteogenic differentiation of BMMSCs has also been demonstrated previously by the addition of sex steroids to BMMSC cultures of healthy rats that showed an increase in mineralized matrix synthesis (10).

The injection of BMMSCs from healthy young rats into the bone marrows of rats with osteoporosis can increase the bone mass. This treatment can be a viable treatment alternative for sites more vulnerable to bone fractures (20). There is the possibility of rejection (33), and therefore, the use of the patient's own cells would be ideal. However, the use of the BMMSCs of patients with osteoporosis would only be a viable alternative if the osteogenic potential of these cells was also recovered *in vitro* prior to inoculation. There are few studies on the biology of BMMSCs of rats with osteoporosis. It has been shown that BMP-2, BMP-7, parathyroid hormone (PTH) and platelet-derived growth factor (PDGF) increase the alkaline phosphatase activity and the concentration of calcium in BMMSC cultures from rat with osteoporosis (23), and that BMMSCs of OVX´d rats respond positively to treatment with estrogen by increases in protein synthesis, alkaline phosphatase activity and collagen I expression (35). However, our study is believed to be the first to verify the effect of addition of T3 on the *in vitro* osteogenic differentiation of BMMSCs of rats with osteoporosis. Previously, only *in vivo* results had shown

that treatment with thyroxine on the skeleton of ovariectomized female rats can reverse osteoporosis after ovariectomy, depending on the dose (7) and disease course (29).

The addition of T3 provides the best results compared with the BMMSCs of rats with osteoporosis without treatment in at least one of the evaluated time points. The cells of rats with osteoporosis treated with T3 (1 nM) showed increased expression of collagen I, osteopontin and BMP-2. Previous studies have demonstrated the beneficial effect of T3 on the osteogenic differentiation of BMMSCs, but only those of young animals and those without metabolic disease (3).

The results demonstrated a positive effect of the addition of T3 on some parameters involved in the osteogenic differentiation of BMMSCs. No dose of T3 caused any adverse effect on the expression of collagen I or on the synthesis of the mineralized matrix in the BMMSCs of rats with osteoporosis. However, further studies on the addition of T3 into BMMSCs cultures of human patients with osteoporosis need to be performed to validate the use of this hormone in BMMSCs cultures. It is also important to verify whether the local treatment of osteoporosis with BMMSCs treated *in vitro* with T3 will have the same success compared with the treatment of BMMSCs of young rats (20). It is also important to search for other sources of MSCs that have not suffered reduction of the osteogenic potential in patients with osteoporosis, as this could be a viable alternative for the treatment of osteoporosis caused by the bilateral ovariectomy or menopause.

According of this study, the T3 did not have a dose dependent effect, in agreement with Milne et al. (15). Not necessarily the same dose of T3 will stimulate the same way the different parameters. T3 shows dose-dependent effect on BMMSCs of young animals, in agreement with Boeloni et al. (3), and T3 shows dose-dependent effect on osteoblasts, in agreement with Ishida et al. (11), Pepene et al. (22). However, in this study, T3 had no dose-dependent effect on BMMSCs of adult animals with osteoporosis. Thus the dose-dependent effect of T3 can vary with the cellular type studied.

Furthermore, interestingly, the effect of addition of T3 in the various culture parameters evaluated did not follow a classical dose-response curve (no monotonic). A dose-response curve is no monotonic when the slope of the curve changes within the range of doses studied. These curves are often U-shape (with maximal responses of the measured endpoint observed at low and high doses) or inverted U-shaped (with maximal responses observed at intermediate doses) as curves observed in some parameters evaluated. Examples of no monotonic dose-response curve are observed in cell culture, in animal models and in epidemiological studies. Several hormones, in addition to estrogens and androgens, may produce no monotonic responses, suggesting that this is a general feature of hormones (32).

Various mechanisms by which hormones produce no monotonic responses in cells, tissues and animals have been identified. These mechanisms are related to cell type, the presence of specific receptors and cofactors, regulation and desensitization of the receptor and the receptor affinity, among others. The receptor affinity can determine that the U-shaped curves can occur. At low doses, certain hormones bind almost exclusively a single receptor or receptor family, but high doses may also bind weakly to multiple hormone receptors (32).

Some researchers suggest that no monotonic curves are artifacts of cell culture, however, a large number of these curves is observed in animal models following the administration of hormones, rejecting the hypothesis that this is a phenomenon observed only in cell culture. The mechanisms responsible for these phenomena *in vivo* may be similar to those found in cell culture systems, although additional mechanisms may be operating *in vivo*, such as the positive and negative feedbacks of the endocrine system (32).

Conclusion

It was concluded that among the studied doses of T3, 0.01 and 1000 nM had a positive effect promoted by increased osteogenic matrix synthesis and collagen expression in at least one of the evaluated time points compared to BMMSCs of rats with osteoporosis without treatment. However, treatment with T3 was unable to reach the osteogenic potential of the MSCs of healthy young rats.

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