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Dose-Dependent Effects of Triiodothyronine on the Osteogenic Differentiation of Rat Bone Marrow Mesenchymal Stem Cells

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Key Words
Mesenchymal stem cells · Triiodothyronine · Osteogenic differentiation · Rats, female

Abstract
Background/Aims: The aim of this study was to investigate the dose-dependent effects of triiodothyronine (T3) on the osteogenic differentiation of mesenchymal stem cells (MSCs). ***Methods:*** MSCs that express CD73, CD54 (intercellular adhesion molecule-1) and CD90 were cultured in triplicate (1×10^5/well) in osteogenic medium with T3 (1, 10, 10^3 or 10^5 pM) or without T3 (control) for 7, 14 and 21 days. Alkaline phosphatase activity, conversion of MTT into formazan crystals, collagen synthesis, collagen maturation, the number of mineralized nodules and their diameters were all determined, and the means were compared by the Student-Newman-Keuls test. ***Results:*** A dose of 10^5 pM T3 resulted in a negative effect on MSC osteogenic differentiation, with less collagen synthesis. The 1 pM T3 dose resulted in greater collagen synthesis and alkaline phosphatase activity and more mineralized nodules than in the control group, similar to the 10 pM dose. Nevertheless, the 10 pM dose demonstrated better results than the 1 pM dose with regard to MSC osteogenic differentiation, with greater MTT reduction, better collagen maturation and a larger mean diameter of mineralized nodules. ***Conclusions:*** The effect of T3 on MSC differentiation is dose-dependent, with the 10 pM dose promoting better bone marrow MSC osteogenic differentiation.

Introduction

The thyroid hormones, represented by thyroxine and triiodothyronine (T3), are essential for the growth and development of many organs and tissues in utero, including bone tissue [1]. In postnatal life, the action of thyroid hormones is essential for the growth, differentiation and metabolic control of many organs [1]. The effects of these thyroid hormones are mainly due to the binding of T3 to the receptors TRα and TRβ, which are present in the cells of many tissues [1, 2], including osteoblasts [3] and mesenchymal stem cells (MSCs) [3, 4].

It is well known that the proliferation and differentiation of MSCs are under the command of many factors. Those factors are internal and external cellular signals represented by products of gene expression (c-fos), cytokines, growth factors, cellular density, physical contact with neighboring cells, physical and mechanical stimuli and hormonal factors [5, 6].

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Despite the diversity of studies that have used stem cells in the treatment of many diseases, there are many aspects of MSC biology that are still ignored. An example of this is the lack of knowledge regarding the effects of thyroid hormones on MSC osteogenic differentiation. In spite of knowledge of the existence of receptors for these thyroid hormones in MSCs [3, 4], only one study in the consulted literature has demonstrated that thyroid hormones increase the expression of mRNA for insulin-like growth factor-1 in MSCs. However, this study does not mention the dose-dependent effect of thyroid hormones [7]. In contrast to the present study, the aim of the study by Milne et al. [7] was to verify the divergent effects of T3 on the osteogenic differentiation of bone marrow cell cultures derived from two different bone sites, i.e. the femurs and vertebrae of rats, indicating site-dependent effects of T3.

Ishida et al. [8] have already evaluated the effect of T3 in regulating the differentiation of rat calvaria cells, which are differentiated cells, unlike the cells used in this study, which were undifferentiated cells submitted to osteogenic differentiation. Thus, until the present study, there was no research on the dose-dependent effect of T3 on the osteogenic differentiation of MSCs, and the present study complements the previous studies.

Various studies in vivo have demonstrated the existence of dose- and time-dependent effects of thyroid hormones on bone tissue. Some studies have demonstrated that, in hyperthyroidism, osteopenia is caused by an increase in bone resorption [9–12]. Based on the results in vivo, our hypothesis was that the effects of T3 on bone metabolism are also due to a dose-dependent effect on the osteogenic differentiation of MSCs. The aim of this study was to verify the dose-dependent effects of T3 on the osteogenic differentiation of MSCs extracted from the bone marrow of female rats.

Materials and Methods

Cell Harvesting and Culture

Female rats (30 days old) were used in this study. To obtain bone marrow samples, the rats were sacrificed with an overdose of anesthesia (pentobarbital sodium, 30 mg/kg), as approved by the Ethical Committee of the Universidade Federal de Minas Gerais. The femurs and tibias were dissected away from the attached muscle and connective tissue under aseptic conditions, and the epiphyses were removed. The bone marrow was flushed with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y., USA), and the released cells were suspended 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/5% CO_2 for 3 days. The nonadherent cell population was removed, and the adherent layer was washed once with fresh medium. The culture medium was changed twice a week during culturing.

FACS Analysis

Cells at the fourth passage were harvested with trypsin/EDTA and centrifuged at 1,400 rpm for 10 min. Cells were then resuspended at 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 fluorophore-conjugated secondary antibody for 30 min at 4°C. The samples were analyzed using a FACScan cytometer (Becton Dickinson), and data were analyzed using Cellquest software (Becton Dickinson). The following primary antibodies were used: anti-CD45, anti-CD90, anti-CD73 and anti-CD54 (BD Biosciences, San Jose, Calif., USA).

Osteogenic Differentiation

After the fourth passage, the adherent cells were harvested by treatment with trypsin/EDTA. They were then counted and plated in 6- and 24-well culture plates (1×10^5 cells/well). The cells 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 (all from Merck, Germany) and 10 nM dexamethasone (Sigma-Aldrich, St. Louis, Mo., USA). The cells were grown at 37°C and 5% CO_2 for 7, 14 and 21 days with different doses of 3,3′,5-triiodo-L-thyronine (T3; 10^5, 10^3, 10 and 1 pM; Sigma-Aldrich). The doses of T3 were added to the culture medium with each change in medium for all experimental periods. For each period, 1×10^5 cells/well without T3 were used as the control group. Thus, there was a total of 5 experimental groups: 10^5 pM T3, 10^3 pM T3, 10 pM T3, 1 pM T3 and control (without T3). The cells were grown for 7, 14 and 21 days to assess the conversion of MTT into formazan crystals, alkaline phosphatase activity, collagen production and maturation and production of mineralized nodules.

MTT Reduction Assay

Briefly, the cells were incubated with MTT for 2 h at 37°C. After incubation with MTT, the cells were treated for 12 h with a solubilization solution (SDS in 10% HCl), and the absorbance at 595 nm for the solubilized MTT formazan product was measured using a microtiter plate reader. The mean absorbance and standard deviation (SD) were determined in triplicate for each experimental group.

Alkaline Phosphatase Activity

In this assay, the chromogens 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) react with alkaline phosphatase, and BCIP is hydrolyzed by alkaline phosphatase to form a blue intermediate. The intermediate is then oxidized by NBT to produce a dimer, which is an intense purple dye [13, 14]. Thus, this assay is an indirect method of verifying alkaline phosphatase activity. Briefly, the cells were incubated with the BCIP/NBT solution (Gibco) for 2 h at 37°C. After incubation, the cells were treated for 12 h with the solubilization solution (SDS

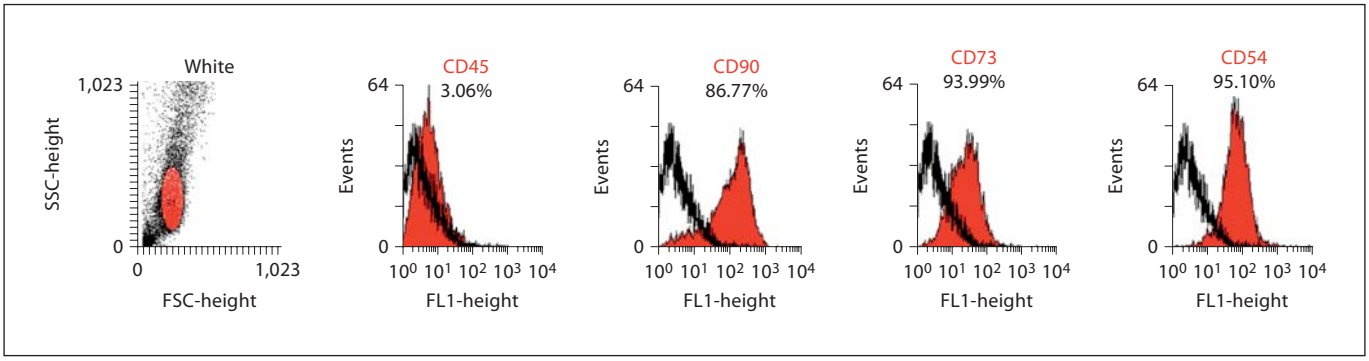

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Fig. 1. Flow cytometric evaluation of CD45, CD90, CD73 and CD54 (ICAM-1) expression frequencies in the MSCs of rats. The fluorescence scale is on the x-axis and is considered positive when the cell peak is above 10^1. The unshaded peak represents unmarked stem cells (negative control). The shaded peak represents stem cells marked with antibodies to CD45, CD90, CD73 and CD54 (ICAM-1).

in 10% HCl). The absorbance at 595 nm was measured using a microtiter plate reader, and the mean absorbance and SD were determined in triplicate for each experimental group.

Collagen Production Assay

The cultures were washed in PBS and fixed in Bouin's fixative solution for 2 h at 37°C. After incubation, the cells were washed with deionized water and then stained with Sirius red (0.1%) for 30 min at room temperature. The culture plates were washed with a 0.01 N HCl solution, air dried and then examined by light microscopy. They were then treated with a 0.5 N NaOH solution, and the absorbance at 540 nm was measured using a microtiter plate reader. The mean absorbance and SD were determined in triplicate for each experimental group.

Picrosirius-Polarization Staining Method

In brief, the cells were washed in PBS and fixed in Bouin's fixative for 2 h at 37°C. After incubation, the cells were washed with deionized water and then stained with Sirius red for 30 min at room temperature. Thereafter, the cell culture plates were washed with a 0.01 N HCl solution, air dried and stained with Sirius red. Next, the cell culture plates were fixed in 70% alcohol at room temperature and examined by polarized light microscopy according to the methods of Junqueira et al. [15].

Hematoxylin and Eosin Staining

The cells were cultured in 6-well culture plates with coverslips (22 × 22 mm). In brief, the cells were fixed in 70% alcohol at room temperature and stained with hematoxylin and eosin according to the methods of Prophet et al. [16]. The number of cells was quantified in 50 fields using a ×20 objective. The mean and SD were then determined in triplicate for each group.

Mineralization Assay

The cells were cultured in 6-well culture plates with coverslips (22 × 22 mm). The cultures 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, after which calcium deposits were stained dark brown. The cells were counterstained with eosin. Thereafter, the number and medium diameter (in micrometers) of the mineralized nodules were assessed by light microscopy. The number of nodules was quantified in 35 fields using a ×10 objective, and the diameter of all the nodules was measured using an ocular micrometer and a ×20 objective. The longitudinal and transverse lengths were measured, and the mean was obtained. The obtained values were converted to micrometers with an ocular micrometer scale. Triplicate experiments were used to determine the mean ± SD.

MitoTracker Green Staining and Flow Cytometry Analysis

MitoTracker Green (MTG) fluorescence has been used as a measure of mitochondrial mass [17]. To study the mitochondrial mass, the stem cells were analyzed after 21 days of culture with DMEM medium, either with T3 (10 pM) or without it (control). The adherent stem cells were washed with trypsin and collected by centrifugation (1,400 rpm at 10 min). The cell pellet was resuspended in DMEM, and cells were stained in 96-well U-bottomed plates with MTG dye diluted 1:10 in 0.15 M PBS, pH 7.0, and incubated for 30 min at 4°C. We used unmarked stem cells as a control. All of the stained cells were analyzed in a FACScan flow cytometer (Becton Dickinson Immunocytometry System, San Jose, Calif., USA), for which the instrument setup was performed using Calibrite beads. A minimum of 10,000 events were acquired for each surface marker. Cellquest software was used for the acquisition of data, and the data were analyzed by the WinMDI program using histogram graphics.

Statistical Analysis

Delineation was entirely at random using a 5 × 3 factorial (5 groups × 3 periods). The data were subjected 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, Calif., USA). Differences were considered significant at $p < 0.05$.

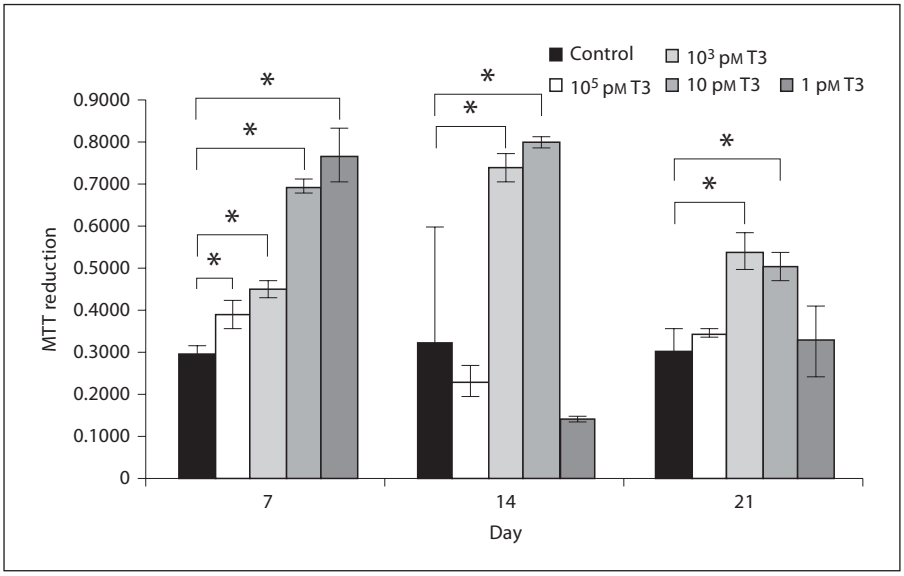

Fig. 2. MTT reduction into formazan crystals (measured by mean ± SD absorbance at 595 nm) in bone marrow MSCs treated with T3 (10^5, 10^3, 10 or 1 pM) or without T3 (control) in osteogenic medium. * $p < 0.05$ compared to the control group.

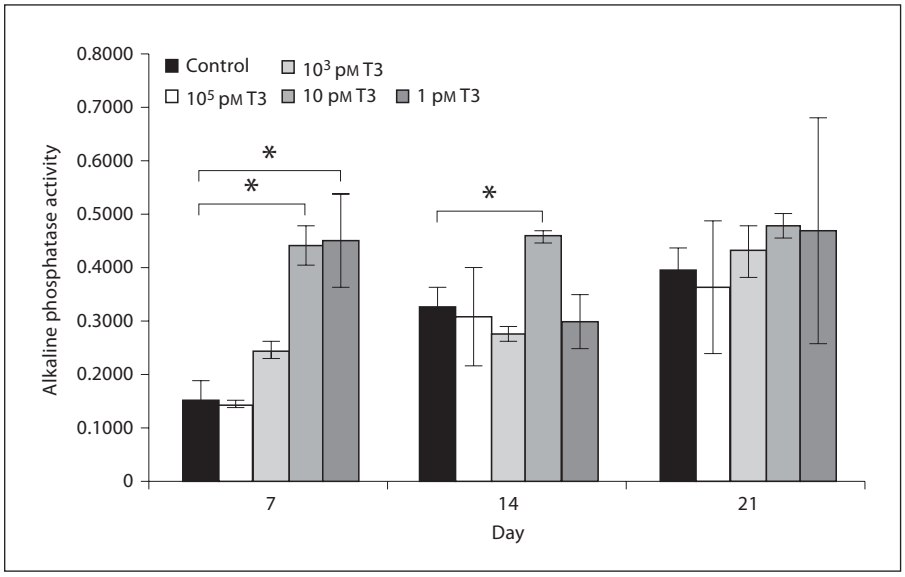

Fig. 3. Alkaline phosphatase activity (measured by mean ± SD absorbance at 595 nm) of bone marrow MSCs treated with T3 (10^5, 10^3, 10 or 1 pM) or without T3 (control) in osteogenic medium. * $p < 0.05$ compared to the control group.

Results

Phenotypic Characterization of the Bone Marrow MSCs

The phenotypic characterization of the bone marrow MSCs indicated that there was no CD45 expression in 96.94% of the cells. There was, however, expression of CD73, CD54 (intercellular adhesion molecule-1; ICAM-1) and CD90 in 93.99, 95.10 and 86.77% of the cells, respectively (fig. 1).

MTT Conversion into Formazan Crystals

At day 7, independent of the dose used (10^5, 10^3, 10 or 1 pM T3), all of the groups treated with T3 presented high capacities for the conversion of MTT into formazan crystals, in comparison to the control group. However, at days 14 and 21, only the doses of 10^3 and 10 pM remained superior to the control (fig. 2).

Alkaline Phosphatase Activity

At day 7, there was an increase in the alkaline phosphatase activity of the groups treated with doses of 10 and

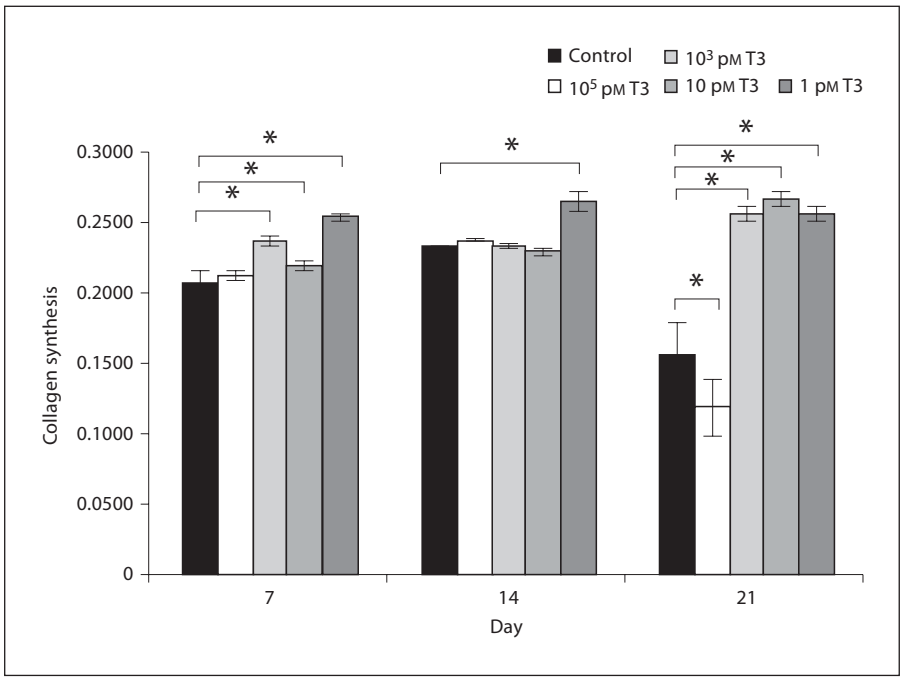

Fig. 4. Collagen synthesis (measured by mean ± SD absorbance at 595 nm) in bone marrow MSCs treated with T3 (10^5, 10^3, 10 or 1 pM) or without T3 (control) in osteogenic medium. * $p < 0.05$ compared to the control group.

1 pM T3 compared to the control and the other groups treated with T3. At day 14, the dose of 10 pM T3 showed a high alkaline phosphatase activity compared to the other evaluated groups. However, at day 21, the alkaline phosphatase activity was similar in all the groups treated with T3 as well as the control (fig. 3).

Collagen Synthesis and Maturation

At day 7, the groups treated with doses of 10^3, 10 or 1 pM T3 showed higher collagen synthesis than the control group. At day 14, only the cells treated with 1 pM T3 showed higher collagen synthesis than the control group. However, at day 21, in the groups treated with doses of 10^3, 10 or 1 pM T3, the collagen synthesis was higher than that in the control group. A negative effect on collagen synthesis was observed at day 21 only at the dose of 10^5 pM T3, in comparison to the control (fig. 4).

For picrosirius-polarization staining at day 21 in culture, the group treated with 10 pM T3 displayed a large amount of collagen and predominantly presented red or yellow (type I) collagen fibers between smaller amounts of green collagen fibers (type III). Furthermore, in comparison with those of the control group as well as the other groups treated with T3, these collagen fibers presented larger birefringence, were thick and were densely distributed in a net-like form.

Interestingly, the group treated with the dose of 1 pM T3, despite having presented higher collagen synthesis in relation to the control at day 21 in culture (fig. 4), presented red collagen fibers with less birefringence than the control group (fig. 5).

Number and Diameter of Mineralized Nodules

At days 7 and 14 in culture, the largest numbers of mineralized brown or black nodules were found at a dose of 10^3 pM T3; however, at day 21, only the group treated with 10 pM T3 showed a significantly higher number of mineralized nodules than the control group (fig. 6).

Regarding the mean diameters of the mineralized nodules, there was not a significant difference between the groups at day 7 in culture. However, at days 14 and 21, the mean diameters were significantly higher in the group treated with 10 pM T3 than in the other groups (fig. 7).

Number of Cells per Field

Although the initial number of cells had been standardized (1×10^5 cells/well), the final number of cells/field varied among the doses of T3 and the culture periods. The dose of 10 pM T3 was the only dose that produced a smaller number of cells for all evaluated periods when compared to the control. The dose of 1 pM T3 was the only dose that did not alter the number of cells for all

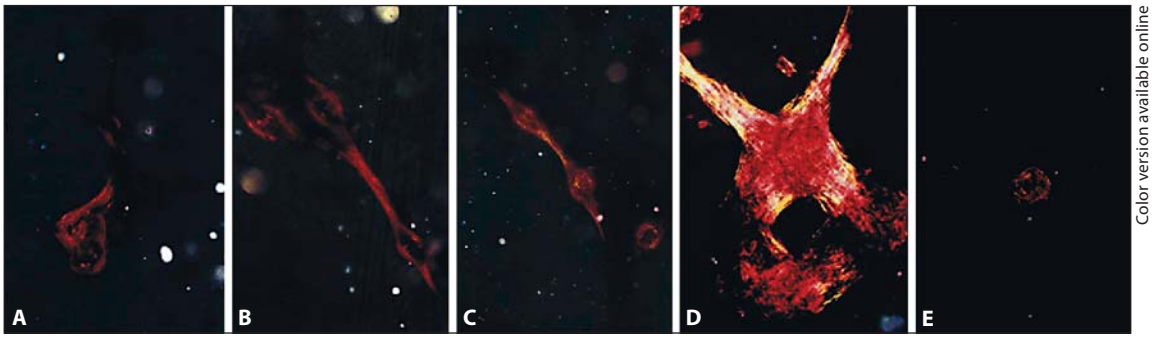

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Fig. 5. Cultures of bone marrow MSCs of female rats at day 21 in osteogenic differentiation medium, with picrosirius-polarization staining by polarized light microscopy (×62.5). **A** Control (without T3). **B** 10^5 pM T3. **C** 10^3 pM T3. **D** 10 pM T3. **E** 1 pM T3. The cells treated with 10 pM T3 (**D**) had denser and more birefringent collagen fibers in comparison with the other groups, and they were densely distributed in a net-like form. The cells treated with 1 pM T3 (**E**) had less birefringent and less dense fibers than the control group.

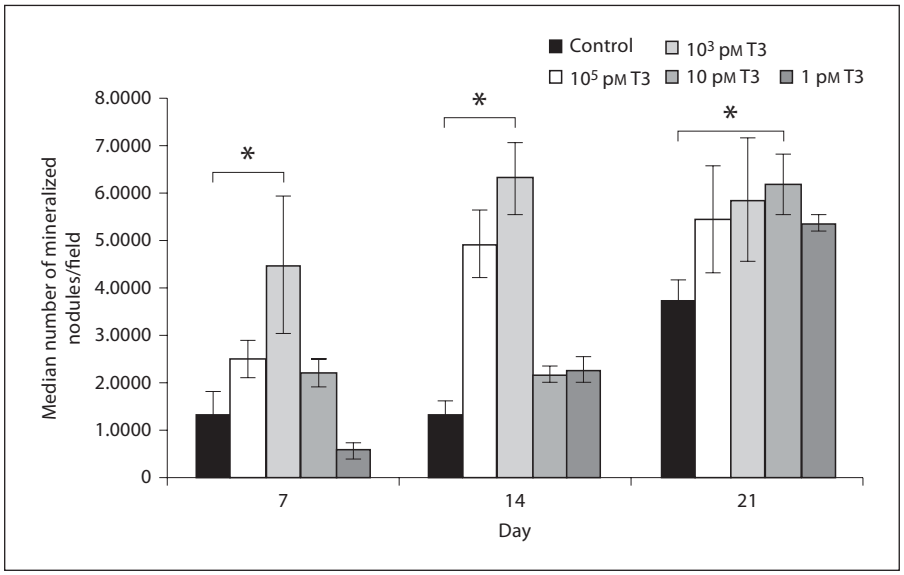

Fig. 6. The median number of mineralized nodules per field (counted with a ×10 objective; mean ± SD) produced by bone marrow MSCs treated with T3 (10^5, 10^3, 10 or 1 pM) or without T3 (control) in osteogenic medium. * $p < 0.05$ compared to the control group.

evaluated periods in relation to the control. In addition, at day 7, the doses of 10^5 and 10^3 pM T3 significantly decreased the percentage of cells when compared to the control. At day 21, the dose of 10^5 pM T3 significantly decreased the percentage of cells when compared to the control (fig. 8).

Mitochondrial Mass

The mitochondrial mass of the MSCs in the control group and the group treated with 10 pM T3 were similar at day 21 of culture with the osteogenic differentiation medium. These groups presented similar percentages of marked cells, indicating a dislocated M1 (shown as a dislocation of the fluorescence positive when the cell peak is above 10^2) as illustrated in the histogram graphic (fig. 9).

Discussion

Bone marrow contains MSCs, hematopoietic cells [5] and fibroblasts [18]. We used the antibodies CD45, CD73, CD90 and CD54 (ICAM-1) for the phenotypic characterization of MSCs. The Mesenchymal and Tissue Stem Cell

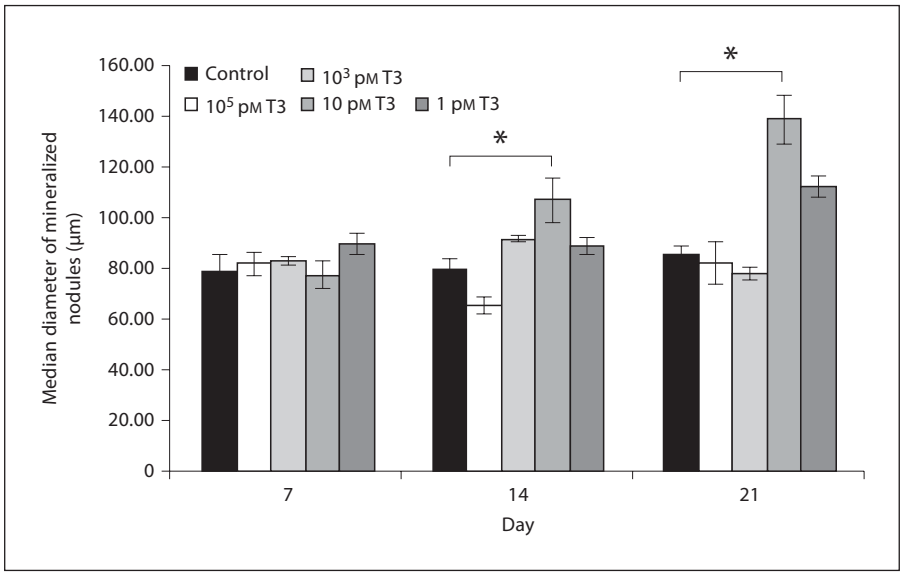

Fig. 7. Median diameter of the mineralized nodules (mean ± SD) produced by bone marrow MSCs treated with T3 (10^5, 10^3, 10 or 1 pM) or without T3 (control) in osteogenic medium. * $p < 0.05$ compared to the control group.

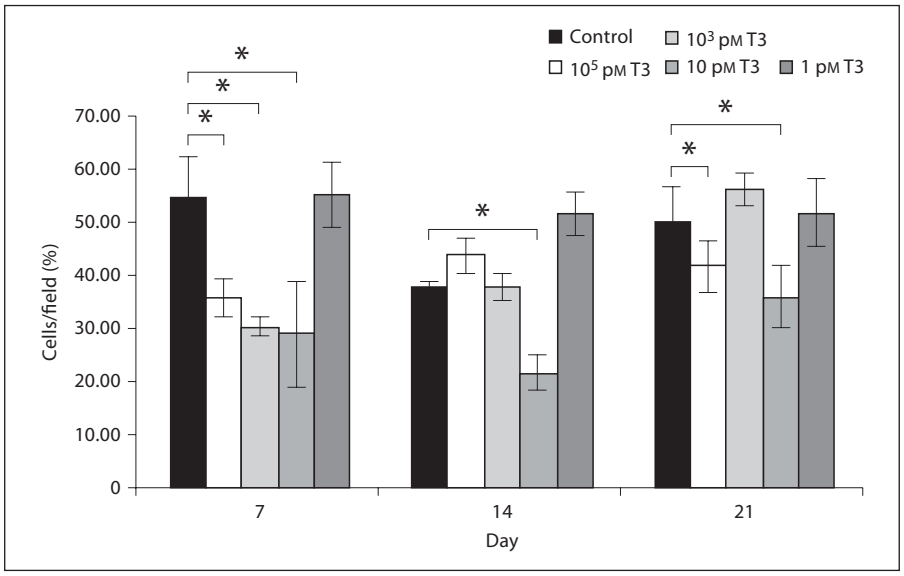

Fig. 8. Percentage of MSCs/field (mean ± SD) in groups treated with T3 (10^5, 10^3, 10 or 1 pM) or without T3 (control) in osteogenic medium. * $p < 0.05$ compared to the control group.

Committee of the International Society for Cellular Therapy has proposed that MSCs must express CD73 and CD90 and not CD45 [19]. Hematopoietic cells express CD45 [5], which can also be expressed in fibroblasts [20]. CD54 (ICAM-1) is not exclusive to MSCs and is expressed in low concentrations in leukocytes [21–23] and endothelial cells [21, 24]. In spite of this, leukocytes and other hematopoietic cells are not plastic-adherent cells [6]. Additionally, only MSCs demonstrate the capacity for osteogenic differentiation [5, 6, 25, 26].

To characterize the osteogenic differentiation of MSCs, the following parameters were assessed: alkaline phosphatase activity, collagen synthesis and the production of mineralized nodules. The parameters used in this study to demonstrate osteogenic differentiation have been frequently used by other researchers [27–29].

The alkaline phosphatase activity in the groups of cells treated with 10 and 1 pM T3 was higher than in the other groups at day 7. As an early marker of osteogenic differentiation [6], the increase in alkaline phosphatase in these groups suggests the occurrence of early osteogenic dif-

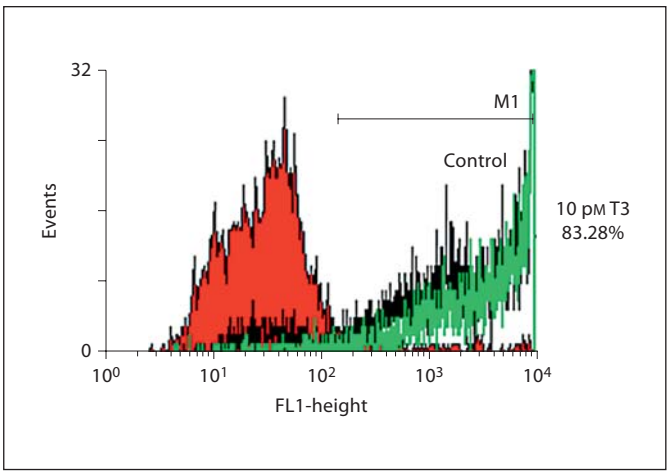

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Fig. 9. Analysis of mitochondrial mass in cultures of female rat bone marrow MSCs cultured in osteogenic differentiation medium at day 21, for groups treated with 10 pM T3 and without T3 (control). The histograms show the fluorescence scale on the x-axis, which is considered positive when the cell peak is above 10^1. The shaded peak represents MSCs that are unmarked (white). The unshaded peak represents MSCs cultured in osteogenic differentiation medium without T3 (control) and MSCs cultured in osteogenic differentiation medium treated with 10 pM T3 marked with MTG (10 pM T3). M1 shows a dislocation of the fluorescence positive when the cell peak is above 10^2. This dislocation was similar between the group treated with 10 pM T3 and control.

ferentiation in comparison with other doses of T3. However, in cultured osteoblasts, 1 and 10 nM T3 (i.e. 10^3 and 10^4 pM, respectively) reduced the alkaline phosphatase activity [30], and the opposite effect was found at 100 nM (i.e. 10^5 pM T3) [31, 32].

Among the doses of T3 studied here, 1 pM induced collagen synthesis and a greater number of mineralized nodules than in the control group at day 21, similarly to the dose of 10 pM. However, MSCs treated with 10 pM T3 demonstrated better osteogenic differentiation and also had higher MTT reduction into formazan crystals, better maturation of collagen and larger diameters for the mineralized nodules.

The dose of 10 pM T3, in addition to stimulating collagen synthesis, also promoted better collagen maturation. However, the dose of 1 pM T3, despite resulting in collagen synthesis similar to the dose of 10 pM T3 and superior to the control group, demonstrated less collagen maturation than other groups, which clearly demonstrates the dose-dependent effect of T3 in bone marrow MSCs. The negative effect of the 10^5 pM T3 dose on the osteogenic differentiation of MSCs was reflected by reduced collagen synthesis in comparison with the control group.

Studies performed with biomaterials such as porous poly(lactide-co-glycolide) have demonstrated that collagen is effective in promoting the adhesion, proliferation and differentiation of bone marrow MSCs of rabbits in association with porous poly(lactide-co-glycolide) [33]. Other results indicate that exogenous type I collagen acts as a component of the intracellular matrix of MSCs and facilitates the osteogenic differentiation and matrix mineralization of MSCs [34]. Thus, it can be suggested that the elevated collagen levels observed following the treatment with 10 pM T3 might provide the best osteogenic potential for MSCs.

Some doses of T3, for at least one of the studied periods, resulted in a reduction in cellular density. The group treated with 10 pM T3 presented less cellular density in all studied periods and a higher conversion of MTT into formazan crystals than the control group, suggesting higher mitochondrial metabolic activity in this group, because mitochondrial succinate dehydrogenase converts MTT into formazan crystals [35]. In addition, considering the effect of T3 on the mitochondrial activity of other cell types [36], as well as the reduction in cellular density in the group treated with 10 pM T3, it was postulated that the higher conversion of MTT into formazan crystals may have occurred as a result of an increase in the number of mitochondria. Therefore, an MTG test was performed to evaluate the mitochondrial mass. The dose of 10 pM T3 was chosen because it promoted a higher conversion of MTT into formazan crystals, while being associated with a reduction in cellular density for all evaluated periods. However, the group treated with 10 pM T3 and the control group presented similar mitochondrial masses. We therefore postulate that the cells of the group treated with 10 pM T3 probably had higher mitochondrial metabolic activity than the control group, mainly due to the activity of succinate dehydrogenase; however, this hypothesis needs to be tested.

Furthermore, it is likely that T3 stimulated collagen synthesis and reduced cellular proliferation, since the group treated with 10 pM T3 had higher alkaline phosphatase activity, collagen synthesis and collagen maturation and greater amounts of mineralized matrix than the other groups. Some studies have confirmed that during osteogenic differentiation there is a proliferative period followed by a differentiation period [37] and that T3 reduces proliferation, stimulating the osteogenic differentiation of pre-osteoblasts [30].

The dose-dependent effect of T3 on the osteogenic differentiation of MSCs in vitro was clearly demonstrated in this study. Interestingly, while a dose of 100 nM T3 (i.e. 10^5 pM T3) stimulated the differentiation of osteoblasts in vitro [30–32], it was not the best dose for promoting the osteogenic differentiation of bone marrow MSCs in female rats.

In conclusion, T3 elicits dose-dependent effects on the osteogenic differentiation of bone marrow MSCs in female rats, with 10 pM T3 promoting the highest level of MSC osteogenic differentiation in vitro. Doses of 0.01–0.1 nM T3 (i.e. 10–10^2 pM) can be considered to be doses at or near physiological concentrations [8]. Thus, the doses of 10^5 and 10^3 pM T3 used in this study are higher than physiological levels. The dose of 10 pM T3 is the dose nearest to physiological concentrations, while 1 nM T3 is lower than the physiological levels.

One limitation of this study is the lack of studies on apoptosis, proliferation and mitochondrial activities of MSCs treated with T3. A further limitation is the fact that we plated an equal number of cells to both 6- and 24-well plates, because this creates much higher cell densities on 24-well plates, and cell densities are crucial in MSC cultures. However, this study might explain the mechanisms by which thyroid hormones produce different dose-dependent effects in vivo. Various studies have demonstrated that patients or animals with hyperthyroidism present osteopenia due to the increase in bone resorption [9–12]; now, from the results of the supraphysiological doses used in this study, it can be suggested that, in hyperthyroidism, the osteopenia might also be due to inhibition of the osteogenic differentiation of MSCs. However, future research in this area is necessary.

Acknowledgments

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