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BIOLOGY

Triiodothyronine does not influence in vitro chondrogenic differentiation of adipose tissue-derived stem cells from young female rat

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ABSTRACT: *The objective of this study was to investigate the in vitro action of triiodothyronine (T3) on the chondrogenic differentiation of adipose tissue-derived stem cells (ASCs) of female rats, with different time periods and doses. ASCs were extracted from female Wistar rats and were cultured in chondrogenic medium with and without the presence of T3. Five groups were established: 1) ASCs without T3; and 2,3,4,5) ASCs with 0.01, 1, 100 and 1,000 nM T3, respectively). After 7, 14 and 21 days, cell morphology, chondrogenic matrix formation, and expression of Sox9, aggrecan, collagen II, and collagen X were evaluated. The Student-Newman-Keuls test was used. ASCs showed CD54, CD73, and CD90 before chondrogenic differentiation. The hormone treatment did not alter chondrogenic matrix formation, Sox9 expression at 14 or 21 days, or expression of collagen II or collagen X at any time. However, the 0.01, 1, and 1000 nM T3 doses decreased Sox9 expression at 7 days. In conclusion, chondrogenic differentiation of ASCs of female rats is not influenced by T3.*
Key words: *Chondrogenesis, thyroid hormone, mesenchymal stem cell, rat.*

Triiodotironina não influencia na diferenciação condrogênica de células tronco do tecido adipose de ratas jovens

RESUMO: *O objetivo do presente trabalho foi verificar o efeito in vitro da triiodotironina (T3) na diferenciação condrogênica de células tronco mesenquimais do tecido adiposo (CTM-TA) de ratas, durante vários períodos e em várias doses. CTM-TA foram coletadas de ratas Wistar e cultivadas em meio condrogênico com ou sem a presença de T3. Constitui-se cinco grupos: 1) CTM-TA sem T3; e 2,3,4,5) CTM-TA com T3 (0,01; 1; 100 e 1000 nM, respectivamente). Após sete, 14 e 21 dias, foram avaliados morfologia celular, formação de matriz condrogênica e expressão de Sox9, agrecano, colágeno II e colágeno X. Para as análises foi utilizado o teste de Student Newman Keuls. CTM-TA expressaram CD54, CD73 e CD90 antes da diferenciação condrogênica. O tratamento hormonal não alterou a formação de matriz condrogênica e a expressão de Sox9 aos 14 e 21 dias e expressão dos colágenos II e X em nenhum dos períodos avaliados. No entanto, as doses de 0,01; 1 e 1000 nM T3 diminuíram a expressão de Sox9 aos 7 dias. Conclui-se que a diferenciação condrogênica de CTM-TA de ratas não é influenciada pela T3.*
Palavras-chave: *Condrogênese, hormônio tireoidiano, célula tronco mesenquimal, rato.*

INTRODUCTION

Studies of stem cells has increased greatly in recent years, especially in relation to their use for the treatment of various illness, like cartilage tissue disorders (LI et al., 2018). Studies have been conducted using stem cells to treat or prevent the progression of cartilage tissue injury, since the progressive degeneration of cartilage inevitably leads to joint pain, swelling and/or stiffness. Cartilage regeneration through stem cell therapy is a promising alternative for the treatment of diseases or defects in

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cartilaginous tissue. However, it is necessary to search for factors that can optimize or increase chondrogenic differentiation of stem cells before *in vivo* treatment.

Mesenchymal stem cells (MSCs) can be obtained from adipose tissue and have the capacity of multipotent differentiation, including chondrogenic (ZUK et al., 2001; DAI et al., 2016). For this reason, MSCs have been widely studied with for the purpose of use in cell therapy. Moreover, the process of obtaining these cells provides a large proportion of MSCs in the total yield of liposuctioned cells, without the need for invasive intervention (FREITAG et al., 2015; DAI et al., 2016).

There are well-defined differentiation protocols to classify adipose tissue-derived stem cells (ASCs) as chondrocytes (ZUK et al., 2001). Nevertheless, many mechanisms and conditions still need to be unraveled, mainly regarding increasing the chondrogenic potential of these cells. Chondrogenic differentiation can be regulated by different conditions (KIM & IM, 2009). In addition, nuclear receptors of thyroid hormones are present in ASCs (ORTEGA et al., 2009). Therefore, these cells are likely to respond to T3 with increased chondrogenic differentiation.

Thyroid hormones influence bone growth *in vivo* by regulating the hypertrophic and maturation zones of the epiphyseal plate, because it controls chondrocyte differentiation (ROBSON et al., 2000). These hormones also stimulate the *in vitro* chondrocyte hypertrophy of embryonic chick cartilage (BURCH & LEBOVITZ, 1982). In cultured mouse tibia, there is an increase in the hypertrophic zone of the epiphyseal plate under the influence of thyroid hormones (MIURA et al., 2002). In addition, thyroid hormones inhibit cell proliferation of ATDC cells (a clonal mouse embryonic carcinoma cell line), and stimulate the chondrogenesis of these cells (SIEBLER et al., 2002). However, there is no research evaluating the potential for chondrogenic differentiation of ASCs of female rats under the influence of thyroid hormones. Thus, the objective of this study was to investigate the *in vitro* action of triiodothyronine (T3) on the chondrogenic differentiation of ASCs of female rats, after different time periods (7, 14, or 21 days) and doses (0.01, 1, 100 or 1,000 nM T3).

MATERIALS AND METHODS

ASC isolation

Euthanasia of four one-month-old female Wistar rats was performed using sodium pentobarbital (30 m/kg). ASCs were extracted according to established protocol (SOLCHAGA et al., 2011). In a surgical environment, the skin and muscle of the ventral abdominal region were cut and the abdominal adipose tissue was harvested. This tissue was placed in culture medium (Dulbecco's modified Eagle's medium; DMEM; Invitrogen, CA, USA) supplemented with penicillin (100 U/mL), gentamicin (60 µg/L), streptomycin (100 µg/mL), and amphotericin (25 µg/L) (Gibco, USA). The adipose tissue was placed in a tube containing collagenase type I (0.15%; Sigma-Aldrich, St. Louis, MO, USA) and placed in an incubator for 60 minutes at 37 °C and 5% CO_2. After incubation, the collagenase was inactivated by the addition of DMEM containing 10% fetal bovine serum (FBS) (LGC Biotecnologia, Brazil). Subsequently, the tube was centrifuged (10 minutes at 1400 *g*) to obtain three phases: fat, red blood cells, and other blood cells and precipitate (stromal phase). The precipitate was placed in DMEM plus 10% FBS and cultivated (37 °C and 5% CO_2) in T75 flasks. All solutions and culture media were prepared with pure water free of microorganisms.

Flow cytometry

When the cells reached the fourth passage and 80 to 90% confluence, their phenotypic characterization was performed by flow cytometry according to previous studies (BOELONI et al., 2014).

Cell viability assay

The ASC viability was tested before chondrogenic differentiation. This evaluation was carried out using Trypan blue, with cells being quantified in a Neubauer chamber, where cells stained blue were considered unviable and transparent cells were considered viable.

Chondrogenic differentiation

Chondrogenic differentiation of ASCs was induced according to previous study (SOLCHAGA et al., 2011). The cells were cultured in chondrogenic medium (StemPro® Chondrogenesis Differentiation Kit; Gibco, USA) with different doses (0.01, 1, 100 or 1,000 nM) of T3 (3,3',5-triiodo-L-thyronine, Sigma-Aldrich) in a pellet system (density of $5x10^5$ cells/15-mL tube), in four replicates, and incubated at 37 °C and 5% CO_2 for 7, 14 or 21 days. After these periods, the expression of Sox9, aggrecan, collagen (Col) II, and Col X was measured. The T3 doses were established according to previous study (BOELONI et al., 2009). with the 0.01 nM dose being close to the physiological dose.

Chondrogenic matrix formation

After 7, 14 or 21 days of differentiation, the pellets were washed with 0.15 M PBS, fixed in 10% buffered formaldehyde for 1 hour and processed by the paraffin-embedded tissue protocol according to previous study (ASSIS et al., 2018). Then the tissues were sectioned (4 µm) and stained with hematoxylin-eosin (HE) for analysis of morphology, or stained with Alcian blue (AB) and periodic acid of Schiff (PAS) for analysis of glycosaminoglycan formation. All analyses were performed by two observers, the positivity scores for AB and PAS were assigned according to the distribution and intensity of the stain, and in accordance with previous study (TRIVILIN et al., 2017).

Total RNA extraction and quantitative PCR (qRT-PCR)

Total RNA extraction was performed using Trizol (Gibco, USA) according to the manufacturer's protocol. For the synthesis of cDNA, 1 µg of RNA was used with the SuperScript III Platinum Two-Step qPCR kit with SYBR Green (Invitrogen, USA). The 7500 Fast Real Time PCR System (Applied Biosystems Inc., USA) was used to perform the qRT-PCR reactions. The GAPDH was used as the housekeeping gene. In addition, $2^{-\Delta\Delta Ct}$ was calculated to compare the treated groups with the control group. The primer sequences used were: Sox9 - sense 5'-CTGAAGGGCTACGACTGGAC -3', antisense 5'-TACTGGTCTGCCAGCTTCCT-3'; aggrecan - sense 5'- CACACGCTACACACTGGACT -3', antisense5'-TCACACTGGTGGAAGCCATC-3',Col II - sense 5'-AGGGGTACCAGGTTCTCCATC-3', antisense 5'-CTGCTCATCGCCGCGGTCCGA-3'; Col X - sense 5'-TGGGTAGGCCTGTATAAAGAACGG-3, antisense 5'-CATGGGAGCCACTAGGAATCCTGAGA-3'; and GAPDH - sense 5'-CAACTCCCTCAAGATTGTCAGCAA-3', antisense 5'-GGCATGGACTGTGGTCATGA-3'.

Statistical analysis

The data were submitted to analysis of variance (ANOVA), and the means were compared by Student-Newman-Keuls (SNK) test (GraphPad Prism 7.000 software). Differences were considered significant if $P < 0.05$.

RESULTS

Phenotypic characterization of ASCs

Regarding phenotypic characterization, there was negative expression for CD45 in 3.04% of the cells and positive expression for CD54 in 91.90%, CD73 in 99.24%, and CD90 in 90.26% of the cells.

Cell viability assay

The viability of the ASCs was greater than 90% before starting chondrogenic differentiation.

Morphological analysis of cells by HE staining

At seven days, the cells were round or oval to elongated, and the nuclei were oval to elongated, displaced to the periphery with predominantly condensed chromatin in the control group. Rare binucleated cells were also observed. The other groups studied (0.01, 1, and 100 nM T3) showed the same morphological characteristics as the untreated group. In contrast, the ASCs treated with 1,000 nM T3 were round and bulky and the nuclei were oval. The ASCs treated with 0.01, 100, and 1,000 nM T3 had loose chromatin, which was not observed in the control group (Figure 1A-E).

At 14 days, the cells were predominantly round, the nuclei were oval to elongated with loose chromatin, and rare binucleated cells were present in the control group. The cell morphology of the other groups treated with T3 was similar to the untreated group (Figure 1F-G).

Like after 14 days, after 21 days the cells were predominantly round, the nuclei were oval to elongated with loose chromatin, and there were rare binucleated cells in the untreated group. The cellular and nuclear morphology of ASCs treated with 0.01 and 1 nM T3 were similar to the untreated group. However, the ASCs treated with 100 nM exhibited round or oval to elongated cells, unlike the control group (Figure 1K-O).

Chondrogenic matrix formation

Regardless of the periods evaluated (7, 14, and 21 days), all treatments (0.01, 1, 100 and 1,000) showed formation of a chondrogenic matrix (AB^+ and PAS^+) similar to the untreated group ($P > 0.05$) (Figures 2 and 3).

Gene expression

Regarding the expression of Sox9 at seven days of differentiation, the ASCs treated with 0.01, 1, and 1,000 nM T3 showed lower expression than the untreated group ($P < 0.05$), whereas the group treated with 100 nM was similar to the untreated group ($P > 0.05$) (Figure 4A). At 14 and 21 days of differentiation, all groups had similar gene expression ($P > 0.05$) (Figure 4B and 4C).

Regarding the expression of aggrecan, Col II, and Col X, all treatments (0.01, 1, 100 and 1,000)

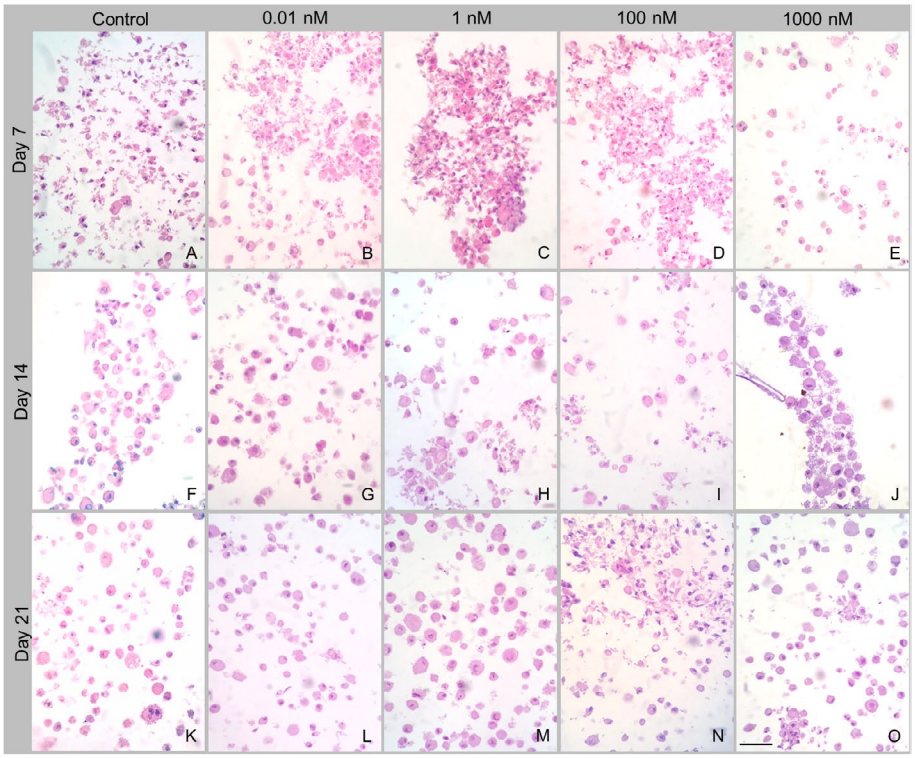

Figure 1 - Morphological analysis of ASCs from female rats treated with T3 (0.01; 1; 100; or 1,000 nM) or not (control) at 7 (A, B, C, D, E), 14 (F, G, H, I, J), and 21 (K, L, M, N, O) days of chondrogenic differentiation. HE staining. Bar = 48.01 µm.

showed the same expression as the untreated group ($P > 0.05$) in all evaluated periods (7, 14, and 21 days) (Figure 4D-L). When the expression of Sox9, aggrecan, Col II, and Col X during the differentiation time was evaluated, all treatments (0.01, 1, 100 and 1,000) showed gene expression similar to the untreated group ($P > 0.05$) (Figure 4).

DISCUSSION

The chondrogenic potential of ASCs has been extensively investigated (GUASTI et al., 2018). However, we believe this study is a pioneer in relation to the action of T3 on the chondrogenic differentiation of ASCs from female rats. T3 did not enhance this differentiation because of decreased Sox9 expression at 7 days of differentiation, and it did not interfere in the expression of aggrecan, Col II or Col X or in the formation of a chondrogenic matrix. This can be explained in part by the results of a previous study demonstrated that thyroid hormone receptors (TRs α and β) are highly active in the absence of exogenous thyroid hormone in human ASCs, and that TRα predominates in multipotent ASC of humans, whereas TRβ is expressed at lower levels and is upregulated during hADSC chondrogenic differentiation (CVORO et al., 2016).

In order to study the chondrogenic potential of ASCs with or without the addition of T3 *in vitro*, we assessed their expression of important genes during differentiation (Sox9, aggrecan, Col II, and Col X) and glycosaminoglycan formation. Regarding the expression of Col X, all T3 treatments stimulated the same gene expression as in the untreated group after all studied intervals (7, 14, and 21 days). In contrast to what was observed in the present study, it is necessary to emphasize that at the beginning of chondrogenesis *in vitro*, cells present a fibroblastoid morphology, and only during this process do they increase in volume (hypertrophy) (STUDER et

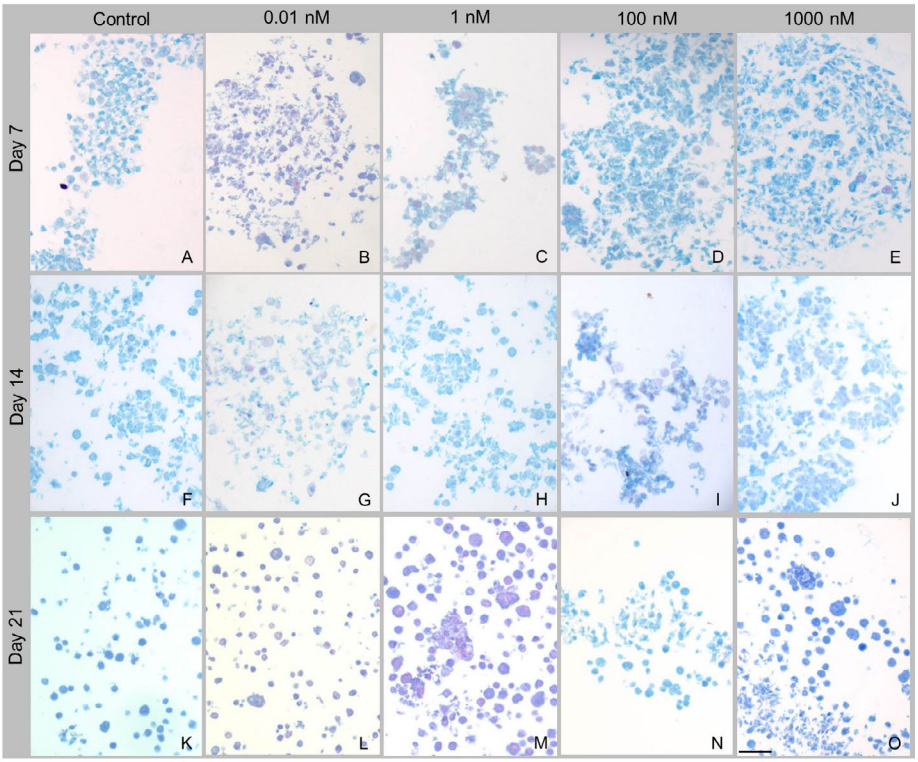

Figure 2 - Formation of AB+ and PAS+ chondrogenic matrix in culture of ASCs from female rats treated with T3 (0.01; 1; 100; or 1,000 nM) or not (control) at 7 (A, B, C, D, E) 14 (F, G, H, I, J), and 21 (K, L, M, N, O) days of chondrogenic differentiation. AB and PAS staining. Bar = 96.02 µm.

al., 2021; LI et al., 2015). *In vivo*, this occurs only at the end of chondrogenesis (SHUM et al., 2003), when hypertrophic chondrocytes express Col X and matrix metalloproteinase (MMP) 13 (KARSENTY & WAGNER, 2002; GOLDRING et al., 2006; CAWSTON & YOUNG, 2010) and programmed cell death occurs, so that the cartilaginous cells can be replaced by bone tissue (DE CROMBRUGGHE et al., 2000; LI et al., 2015).

The chondrocyte hypertrophy may be due to changes in pressure inside and outside the cells, as well as the degeneration of chondrocytes surrounding the extracellular matrix (MACKIE et al., 2011). For this reason, strategies to minimize this hypertrophy have been investigated, such as use of PTHrp, TGF-β1, and MMP13 inhibitor, among others (CHEN et al., 2015). Other factors can also cause hypertrophy during the chondrogenic differentiation of human bone marrow mesenchymal stem cells (BM-MSCs), such as absence of TGF-β combined with dexamethasone (1 nM) and T3 (1 nM) (MUELLER & TUAN, 2008). Moreover, the addition of T3 (1 nM) at 21 and 28 days during human BM-MSC chondrogenesis can induce BMP-4-mediated cell hypertrophy, an effect that is blocked by the BMP antagonist Noggin. In addition to inducing hypertrophy, hormone treatment combined with BMP-4 increases the production of Col II and Col X (KARL et al., 2014). Similar to previous studies, in the present study, hormone treatment (regardless of dose) also caused an apparent cell enlargement, but in this case it did not interfere with chondrogenic matrix formation.

Regarding gene expression, ASCs treated with T3 (0.01, 1, and 1,000 nM) showed less Sox9 expression at 7 days of differentiation. Sox9 plays an important role during chondrogenic differentiation of mesenchymal stem cells (AUGELLO & DE BARI, 2010), being expressed throughout differentiation (KOCH et al., 2000), but not in hypertrophic

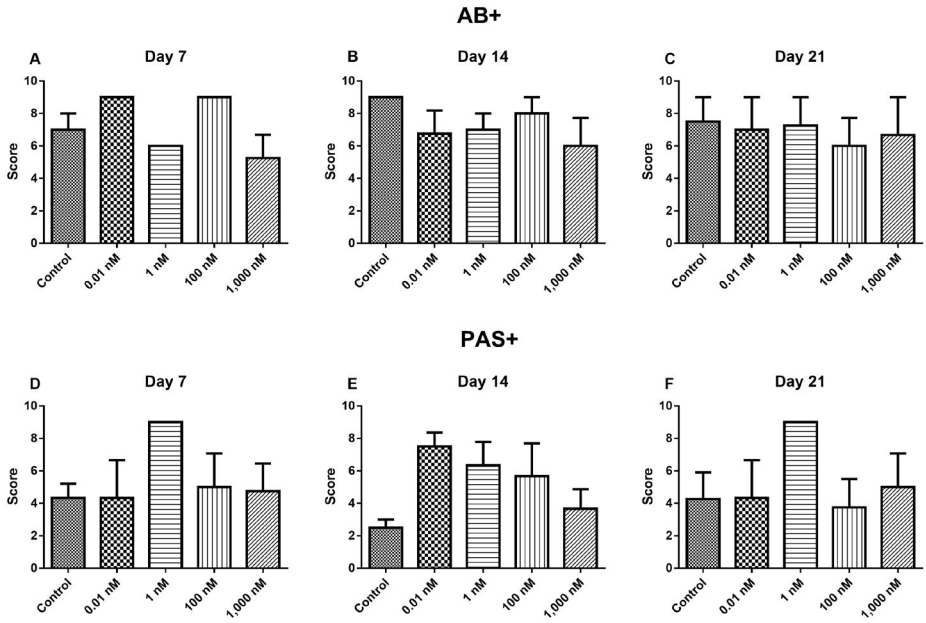

Figure 3 - Production of AB+ chondrogenic matrix (mean ± standard deviation in quadruplicate/group) in ASCs from female rats treated with T3 (0.01, 1, 100, or 1,000 nM) or not (control) at 7 (A), 14 (B), and 21 (C) days of chondrogenic differentiation. Production of PAS+ chondrogenic matrix (mean ± standard deviation in quadruplicate/group) in ASCs from female rats treated with T3 (0.01, 1, 100, or 1,000 nM) or not (control) at 7 (D), 14 (E), and 21 (F) days of chondrogenic differentiation.

chondrocytes (AKIYAMA et al., 2002; DY et al., 2012; LEFEBVRE & DVIR-GINZBERG, 2017). In this context, thyroid hormone (30 ng/mL T4) acts *in vitro* on chondrocytes in the terminal period of differentiation, decreasing the Sox9 expression at 7 days and consequently stimulating cell hypertrophy (OKUBO & REDDI, 2003). These results part agree with what was observed in the present study, but at a different dose and in another cell type. Moreover, treatment with T3 did not modify the Sox9 expression at 14 and 21 days of differentiation.

In our study, the presence of thyroid hormone did not modify the expressions of aggrecan, Col II or Col X in ASCs in any of the evaluated periods. This finding corroborates in part an *in vitro* study that showed that hormone treatment also does not affect expression of aggrecan of BM-MSC (ASSIS et al., 2018), and does not affect chondrogenic matrix percentage of ASC (SPILA et al., 2019). In addition, *in vitro* hormone treatment (30 ng/mL T4) did not alter the Col II expression at 21 days of culture of chondrocytes in the terminal differentiation phase (OKUBO & REDDI, 2003). During *in vivo* chondrogenic differentiation, Col II expression occurs, and as cells differentiate into hypertrophic chondrocytes, its expression decreases while the expression of Col X increases (SHUM et al., 2003). Thus, this whole explanation suggests that both the hormone treatment and the donor source of stem cells affect chondrogenic differentiation. In addition, *in vitro* findings can differ from *in vivo* observations because the interaction of factors occurring *in vivo* is not present in cultures.

CONCLUSION

Hormonal treatment with triiodothyronine does not alter chondrogenic differentiation because less expression of sox9 occurs in a dose dependent manner, and it does not modify aggrecan, Col II or Col X expression or chondrogenic matrix formation.

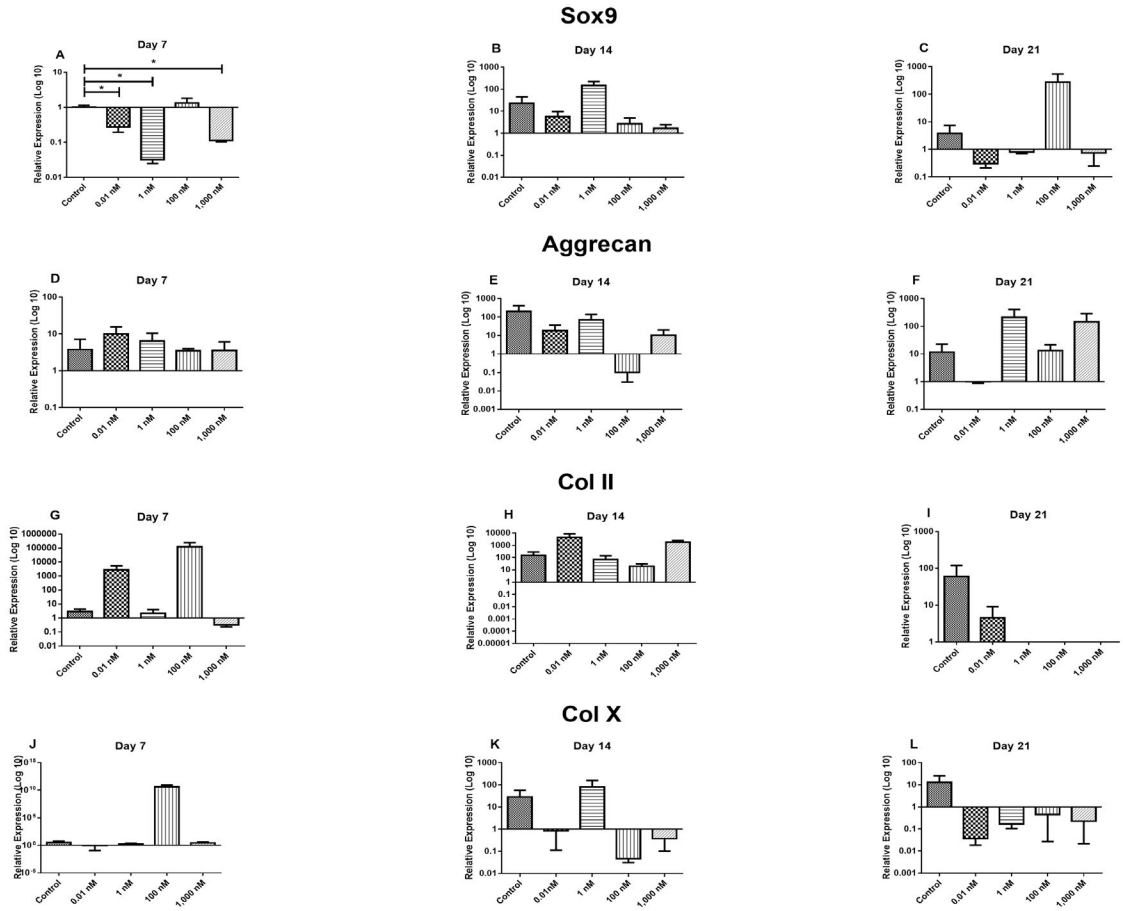

Figure 4 - Relative expression of Sox9, aggrecan, Col II, and Col X (mean ± standard deviation in quadruplicate/group) by qPCR in ASCs from female rats treated or not with T3 at a dose of 0.01, 1, 100, or 1,000 nM, at 7 (A, D, G, J), 14 (B, E, H, K), and 21 days (C, F, I, L) of chondrogenic differentiation. $^{*}P < 0.05$.

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DECLARATION OF CONFLICT OF INTEREST

All authors declare no conflict of interest.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

All experimental procedures with animals were approved by the Ethics Committee in Animal Experimentation, Universidade Federal do Espírito Santo (UFES) (protocol number 062/2013).

AUTHORS' CONTRIBUTIONS

JNB, RS, NMO, AMG and AMAS designed and designed experiments. NCH, HAA, LPR and LPR performed the experiments. The JNB, FPC and GGP supervised the experiments. The NCH and JNB performed statistical analyzes of experimental data. NCH, LPR and JNB prepared the draft manuscript. All authors critically reviewed the manuscript and approved the final version.

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