http://dx.doi.org/10.1590/1678-4162-9364

Arq. Bras. Med. Vet. Zootec., **v.69, n.6, p.1573-1580, 2017**

Osteogenic differentiation of adipose tissue-derived mesenchymal stem cells cultured with different concentrations of prolactin

[*Diferenciação osteogênica de células tronco mesenquimais do tecido adiposo cultivadas com diferentes concentrações de prolactina*]

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ABSTRACT

The objective was to evaluate the *in vitro* effect of prolactin in osteogenic potential of adipose tissue-derived mesenchymal stem cells (ADSCs) in female rats. ADSCs were cultured in osteogenic medium with and without the addition of prolactin and distributed into three groups: 1) ADSCs (control), 2) ADSCs with addition of 100ng/mL of prolactin and 3) ADSCs with addition of 300ng/mL of prolactin. At 21 days of differentiation, the tests of MTT conversion into formazan crystals, percentage of mineralized nodules and cells per field and quantification of genic transcript for alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein, BMP-2 and collagen I by real-time RT-PCR were made. The addition of prolactin reduced the conversion of MTT in group 3 and increased the percentage of cells per field in the groups 2 and 3, however without significantly increasing the percentage of mineralized nodules and the expression of alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein, BMP-2 and collagen I. In conclusion, the addition of prolactin in concentrations of 100ng/mL and 300ng/mL does not change the osteogenic differentiation to the ADSCs of female rats despite increase in the cellularity of the culture.

Keywords: rat, osteogenic differentiation, osteoblasts, hormone, adipose tissue

RESUMO

O objetivo do presente trabalho foi avaliar o efeito in vitro *da prolactina sobre o potencial osteogênico de células-tronco mesenquimais do tecido adiposo (CTM-TA) em ratas. CTM-TA foram cultivadas em meio osteogênico com e sem adição de prolactina e distribuídas em três grupos: 1) CTM-TA (controle), 2) CM-TA com adição de 100ng/mL de prolactina e 3) CTM-TA com adição de 300ng/mL de prolactina. Aos 21 dias de diferenciação, foram realizados os testes de conversão do MTT em cristais de formazan, porcentagem de nódulos mineralizados e células por campo e quantificação dos transcritos gênicos para fosfatase alcalina, osteopontina, osteocalcina, sialoproteína óssea, BMP-2 e colágeno I. A adição de prolactina reduziu a conversão do MTT no grupo 3 e aumentou a porcentagem de células por campo nos grupos 2 e 3, sem alterar significativamente a porcentagem de nódulos mineralizados e a expressão de fosfatase alcalina, osteopontina, osteocalcina, sialoproteína óssea, BMP-2 e colágeno I. Conclui-se que a adição de prolactina nas concentrações de 100ng/mL e 300ng/mL não altera a diferenciação osteogênica das CTM-TA de ratas, apesar do aumento de celularidade da cultura.*

Palavras-chave: rato, diferenciação osteogênica, osteoblastos, hormônio, tecido adiposo

INTRODUCTION

The physiological hyperprolactinemia that occurs during pregnancy and lactation has been associated to bone loss in women and animals (Zuni *et al*., 1999; Coss *et al*., 2000; Dolinska *et al*., 2010), with significant reduction of bone mineral density. The bone mass reduction observed in pregnant women varies from 2.1% to 9.4% in backbone; 0.9% to 3.9% in hip and approximately 2% to 4% in the radius (Karlsson *et al*., 2001). At lactation, bone losses were described between 0.4% and 7.5% in lumbar

Recebido em 22 de agosto de 2016
Aceito em 19 de janeiro de 2017
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vertebrae, 2% to 5% in the femur and 0.2% to 7% in the radius (Karlsson *et al*., 2001; Karlsson *et al*., 2005). Young women with high blood levels of prolactin present bone mineral density similar to women in post-menopause (Alder *et al*., 1998) and individuals with high levels of prolactin, arising from prolactinoma, also show an increase of bone metabolism with consequent bone loss (Colao *et al*., 2000). But, it is probable that the effect of prolactin is dependent of their concentrations and the cell type on which is acting. This is because, contrary to the research where the increase of prolactin is associated to osteopenia (Alder *et al*., 1998; Colao *et al*., 2000), research with cells of osteosarcoma, cells that present receptors for prolactin, cultivated with prolactin demonstrated increase in osteoblastic activity (Bataille-Simoneau *et al*., 1996).

The genesis of osteopenia resulting from hyperprolactinemia has not been completely elucidated. Studies with addition of different concentrations of prolactin in cultures of osteoblasts, cells that express receptors for this hormone (Charoenphandhu *et al*., 2007), have demonstrated conflicting results, and there is either increased expression of genes of osteogenic differentiation such as RUNX-2, alkaline phosphatase, osteocalcin or decrease in the expression of these genes (Charoenphandhu *et al*., 2008; Seriwatanachai *et al*., 2009). Some researchers demonstrate that the bone loss due to increased bone reabsorption, arising from hypersecretion of prolactin, is mediated by estrogen deficiency (Biller *et al*., 1992; Naliato *et al*., 2005). This assertion has already been refuted since women with hyperprolactinemia do not present improvement of bone changes after restoration of gonadal endocrine function (Klibanski *et al*., 1988; Schlechte *et al*., 1992). It is possible that osteopenia originated from excess prolactin is caused by the reduction in the synthesis of bone matrix, since in female rats towards the end of the lactation there is hypoplasia and hypotrophy of osteoblasts (unpublished data). As the osteoblasts are originated from mesenchymal stem cells (MSC), the reduction of its number in the bones of female rats in lactation fosters the hypothesis of this study that the osteopenia, originated from the excess of prolactin, could be result of its effect on the osteogenic differentiation of mesenchymal stem cells.

Multiple studies have already demonstrated the participation of mesenchymal stem cells in the genesis of osteopenia induced by endocrine diseases such as hypo and hyperthyroidism (Boeloni *et al*., 2010; Boeloni *et al*., 2013a; Boeloni *et al*., 2013b; Boeloni *et al*., 2013c; Boeloni *et al*., 2015) and diabetes (Zhao *et al*., 2013; Ha *et al*., 2015). Although receptors for prolactin have already been identified in human mesenchymal stem cells, this seems to be the first study that investigated the effects of prolactin in the osteogenic differentiation of adipose tissue-derived mesenchymal stem cells. Thus, the objective of this study was to evaluate, under the osteogenic differentiation of ADSCs, two concentrations of prolactin that mimic the ones found in the blood of pregnant and lactanting female rats in order to infer on the isolated involvement of this hormone in the ADSCs.

MATERIAL AND METHODS

Three 45-days-old female *Wistar* rats (*Rattus norvegicus*) were used. The rats were euthanized with an overdose of an association of intraperitoneal anesthetic. All described procedures were approved by the Ethics Committee in Animal Experimenting at Universidade Federal de Minas Gerais (UFMG) (protocol 378/2012).

Immediately after the euthanasia, the extraction of ADSCswas performed according to the protocols already established (Zuk *et al*., 2002; Gomide *et al*., 2011). The visceral adipose tissue was collected aseptically in a laminar flow in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y., USA) plus antibiotics and antimycotics (60µg/L gentamicine, 25µg/L amphotericin B, 100U/mL penicillin and 100µg/mL streptomycin, Merck, Germany). The tissue was cut into small fragments, transferred to a tube containing 0.15% collagenase (Collagenase Type I, Gibco, Grand Island, N.Y., USA) diluted in 0.15M phosphate buffer solution (PBS) standard and incubated for 60 minutes at 37°C and 5% CO_2, shaking it every 15 minutes. Afterwards, it was centrifuged for 10 minutes at 1400g. The supernatant was discarded and the precipitate was resuspended in DMEM supplemented with antibiotics, antimycotics and 10% fetal bovine serum and collected in 75-cm^2

culture flasks in an oven at 37°C and 5% CO_2. The culture medium was changed twice a week.
Cells cultured until the third passage in DMEM were trypsinized and centrifuged at 1400g for 10 minutes. The cells were re-suspended at a concentration of 1 x 10^6 cells in PBS and transferred to 96-well plates with primary antibody for 30 minutes at 4ºC. The cells were washed with PBS and incubated with a secondary antibody conjugated with fluorescent (Alexa Fluor 488, Molecular Probes TO, OR, USA) for 30 minutes at 4ºC. The samples were analyzed using a cytometer FACScan (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-CD90 (clone Ox-7 mouse), anti-CD73 (clone 5 F/B9 mouse) and anti-CD54 (clone 1A29 mouse) (BD Biosciences, San Jose, CA, USA).

After the initial culture in DMEM and four passages obtaining confluence of 80 to 90%, the adherent cells were counted and trypsinized, distributed in six and 24 wells and in 25-cm^2 culture flasks (1x 10^4 cells/cm^2). The basic medium was replaced through osteogenic medium, which consisted of DMEM added of antibiotics, antimycotics, 10% of fetal bovine serum plus50µg/mL ascorbic acid, 10mM β-glycerophosphate (Merck, Germany) and 10nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). After the addition of the osteogenic medium, three experimental groups were formed with the addition of different concentrations of prolactin (Prolactin human, Sigma Aldrich, St Louis, MO, USA): 1) ADSCs (control), 2) ADSCs with addition of 100ng/mL of prolactin and 3) ADSCs with addition of 300ng/mL of prolactin. The cells were cultivated in oven with 5% CO_2 at 37ºC. The concentrations of prolactin were determined according to studies carried out by Seriwatanachai *et al* (2009) and Ritchie *et al* (1998) and are values that mimic those achieved during gestation and lactation of female rats respectively. At 21 days of differentiation, the tests of MTT conversion into formazan crystals, percentage of mineralized nodules and cells per field and quantification of alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein, BMP-2 and collagen I by real-time RT-PCR were made.

For each experimental group, 1x10^4 ADSCs/cm^2 was cultivated in 24-well plate with osteogenic medium with and without addition of prolactin. At the end of 21 days, the cultures were submitted to test for the conversion of the MTT {bromide [3-(4,5-dimethylthiazol-2il)-2,5-diphenyl tetrazolium]} in formazan crystals. The medium was replaced by 210 µL of osteogenic medium in each well and 170 µL of MTT (5 mg/mL) (Invitrogen, Carlsbad, CA, USA). The plate was incubated for two hours in the oven at 37°C and 5% CO2. The formazan crystals were observed under the microscope. Before, 210 µL of sodium dodecyl sulphate (SDS) with 10% HCl were added which remained *overnight* in the oven at 37°C and 5% CO_2. Subsequently, 100 µL/well were transferred to 96-well plate for analysis with wavelength of 595nm. The mean and standard deviation were determined for each experimental group. The tests were carried out in triplicate.

Briefly, for each experimental group, 1x10^4 ADSCs/cm^2 was cultivated in 6-well plates with sterile cover slips. After 21 days, the wells were washed in PBS, cove slips fixed in 4% paraformaldehyde for 24 hours and subsequently rinsed in distilled water. After being added the silver nitrate solution at 5% (silver nitrate P.A. LabSynth, São Paulo, Brazil) the wells were exposed to light for two hours. The plates were washed with distilled water and the residual silver nitrate was neutralized by a solution of 5% sodium thiosulphate (sodium thiosulphate P.A. LabSynth, São Paulo, Brazil). The cells were counted and stained with eosin. The percentage of the mineralized nodules and cells per field were assessed by light microcopy and were quantified in 40 fields using an ocular piece containing a 121-point grid (Zeiss KPL, 10x) with a 10x magnification. The mean and standard deviation were determined for each experimental group. The tests were carried out in triplicate.

The quantification of the relative genic expression of alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein, BMP-2, collagen I in the ADSCs was compared between the three experimental groups with 21 days of differentiation. The extraction of total RNA of cells was done in three 25-cm^2 culture flasks per group by using Trizol (Life Technologies, CA, USA). The extraction method consisted of an initial step lysis and homogenization of the cell

monolayer for five minutes at room temperature, for complete decoupling of nucleoprotein complexes. The lysate was transferred to a microtube of 1.5mL and 0.2mL was added with chloroform, followed by 10 seconds of homogenization, three minutes of incubation at room temperature and centrifuged at 12,000g for 15 minutes at 4ºC, for separation into three stages. The aqueous phase was transferred to a new tube, with the addition of 0.5mL of isopropyl alcohol and incubated for 30 minutes at a temperature of -80ºC, followed by centrifugation at 12,000 g for 10 minutes to 4ºC to precipitation of RNA. The pellet was then washed with 1 mL of ethanol 75%, homogenized and centrifuged at 10,500g for five minutes at 4ºC. The RNA was solubilized in DEPC water (water treated with dimethyl pirocarbonate, Invitrogen, Carlsbad, CA, USA) free of RNAse, incubated at 56ºC in a thermoblock for 10 minutes and immediately stored at -80ºC. The RNA concentration of each group was determined by the absorbance reading at 260/280nm by spectrophotometry. Reverse transcription reactions were performed using commercial *kit* (SuperScriptTM III Platinum® Two-Step qRT-PCR Kit with SYBR Green, Invitrogen, CA, USA), being that it was used 1 µg of total RNA for cDNA synthesis with a final volume of 20 µL. It was still performed, the real time PCR reactions using 2µL of cDNA, 5pM of each primer and 12.5µL of reagent syber Green (SuperScriptTM III Platinum® Two-Step qRT-PCR Kit with SYBR Green, Invitrogen, CA, USA) in a final volume of 25µL of reaction, at the equipment *SmartCycler System* (SmartCycler® System, Cepheid, Sunnyvale, CA, USA). The parameters used for amplification were: 50°C for 120 seconds, 95°C for 150 seconds and 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The primers were designed based on the sequencing of the mRNA *Rattus norvegicus* (Table 1). The gene expression, the mean and standard deviation were determined in triplicate for each experimental group using the method $2^{-\Delta\Delta CT}$, in which the results obtained for each group were compared quantitatively after normalization based on the expression of GAPDH *Rattus norvegicus.*

The experimental design was completely randomized. A variance analysis was performed (ANOVA) and, for each variable, the means and the standard deviation were determined. The means were compared by the SNK test (Student-Newman-Keuls) using the *Graphpad Instat* 3.05 (GraphPad Software Inc., San Diego, USA). The changes in the expression measured by real-time RT-PCR were compared by the SNK test. Differences were considered significant if $P<0.05$.

Table 1. List of genes and nucleotide sequence of the primers for RT-PCR in real time

Gene	Primers (5’ - 3’)	Acess
Alkaline phosphatase	*forward*: CTAGTTCCTGGGAGATGGTA *reverse*: GTGTTGTACGTCTTGGAGAGA	NM 013059.1
Osteopontin	*foward*: ATCTCACCATTCCGATGAATCT *reverse*: TCAGTCCATAAGCCAAGCTATCA	AB 001382
Osteocalcin	*foward*: CATCTATGGCACCACCGTTT *reverse*: AGAGAGAGGGAACAGGGAGG	NM 013414.1
Bone sialoprotein	*foward*: TGTCCTTCTGAACGGGTTTC *reverse*: CTTCCCCATACTCAACCGTG	NM 012587.2
BMP-2	*foward*: TAGTGACTTTTGGCC ACGACG *reverse*: GCTTCCGCTGTTTGTGTTTG	NM 017178
Collagen I	*foward*: GCAAGGTGTTGTGCGATGACG *reverse*: GGGAGACCACGAGGACCAGAG	NM 000088
GAPDH	*foward*: CAACTCCCTCAAGATTGTCAGCAA *reverse*: GGCATGGACTGTGGTCATGA	NM 002046

RESULTS

The cells extracted from the adipose tissue showed phenotypic characteristics compatible with mesenchymal stem cells, i.e. 90.26% of cells presented expression for CD90, 99.24% of cells presented expression for CD73, 91.90% of cells presented for CD54 expression and only 3.04% of cells presented for CD45 expression.

The group of ADSCs differentiated with addition of 300ng/mL presented minor conversion of MTT as compared to control. The group 2 presented no difference when compared to the control group (Figure 1).

The addition of prolactin in the osteogenic medium promoted positive effect significantly increasing the percentage of cells in the groups 100ng/mL and 300ng/mL in relation to the control group. However, there was no difference in the percentage of mineralized nodules between the groups that received prolactin and the control group (Figure 1).

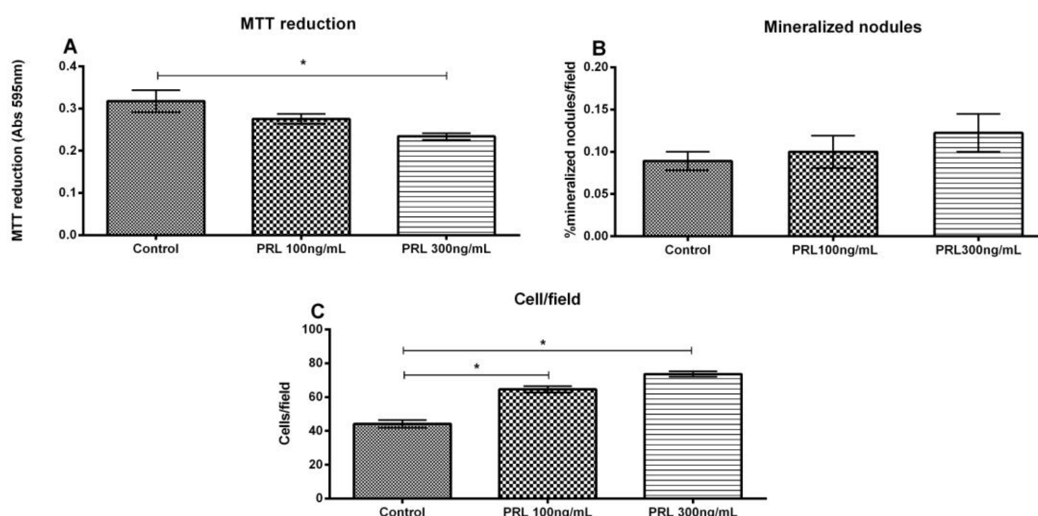

Figure 1. ADSCs cultured in osteogenic medium with and without addition of prolactin after 21 days of differentiation. A) MTT reduction into formazan crystals (mean±SD absorbance at 595nm). B) Percentage of mineralized nodules (mean±SD) and C) percentage of cells per field (mean±SD). *P<0.05.

The addition of prolactin in the osteogenic medium did not alter the relative expression of gene transcripts for alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein, BMP-2, collagen I when compared to the control group (Figure 2).

DISCUSSION

The phenotypical characterization showed that the cells used presented characteristics compatible with the mesenchymal stem cells, i.e., more than 90% of the cells presented expression of surface for CD90, CD73 and CD54 and less than 5% presented marking for CD45 that is expressed by hematopoietic cells. These results comply with what is recommended by International Society for Cellular Therapy assuring that the experiment was performed with culture of stem cells. This characterization is important because the vascular stromal fraction extracted from the adipose tissue contains beyond the ADSCs, other cell types such as endothelial cells, fibroblasts and hematopoietic cells (Dominici *et al*., 2006).

The prolactin in the concentration of 300ng/mL reduced the conversion of MTT of formazan crystals even having increased cellularity, characterized by increasing the percentage of cells per field. The assay of dimethylthiazol (MTT) is a quantitative colorimetric method that is based on the capacity of succinate dehydrogenase, an enzyme active in mitochondria, in converting the water-soluble salts of the tetrazolium. This assay is dependent

of mitochondrial metabolism. The MTT assay has been used to evaluate the cellular viability (Berridge *et al*., 2005). However, in the present study, it can not be said that the concentration of 300ng/mL of the prolactin has reduced the cellular viability, since the percentage of cells/field was significantly higher than control. MTT assay does not determine if the increase in the percentage of cells observed in the ADSCs with addition of 300ng/mL of prolactin was due to increased cell proliferation or decreased apoptosis.

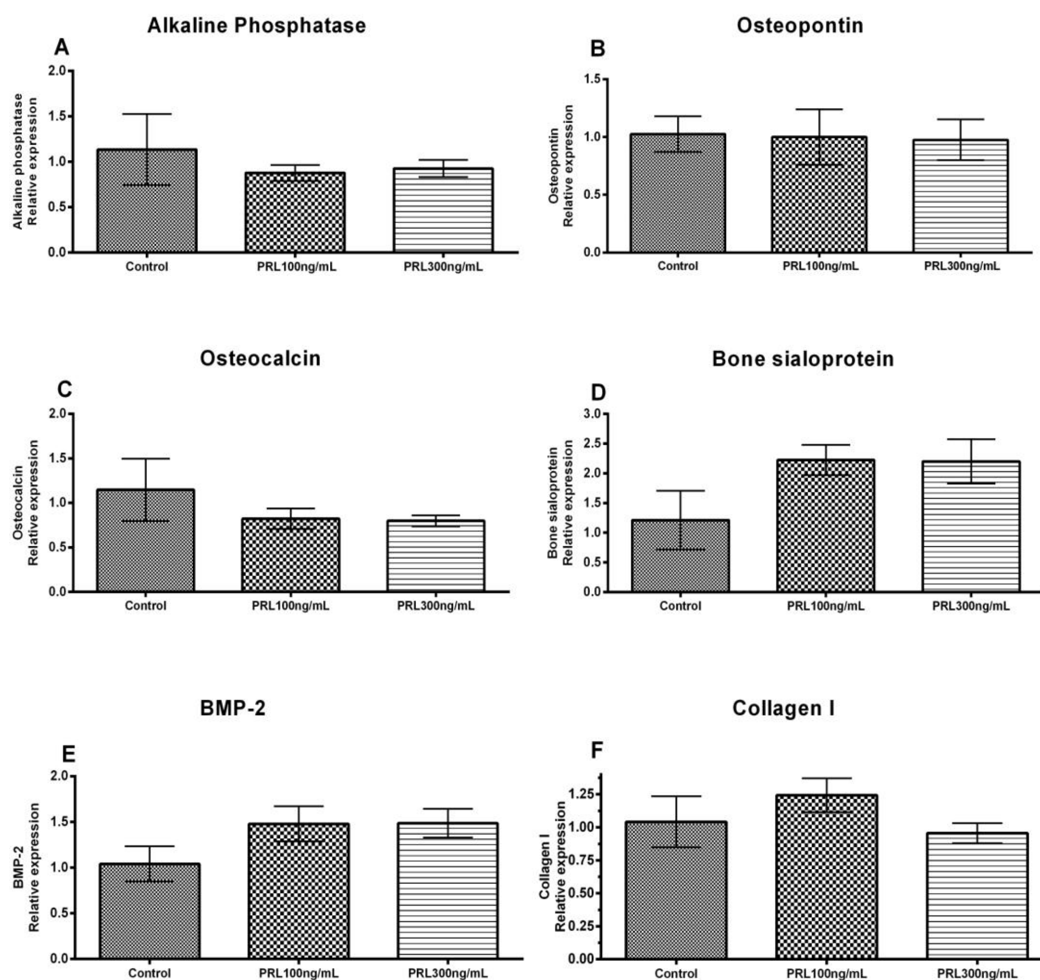

Figure 2. The relative quantification (mean ± SD) of genes expression via real-time RT-PCR of the ADSCs cultured in osteogenic medium with and without addition of prolactin after 21 days of differentiation. *P<0.05. A) Alkaline phosphatase, B) Osteopontin, C) Osteocalcin, D) Bone sialoprotein, (E) BMP-2 and (F) Collagen I.

The addition of prolactin in the culture of ADSCs in the two studied concentrations did not alter the expression of genic transcripts of osteogenic differentiation as well as the formation of the mineralized nodules. More studies are needed to verify if the addition of prolactin *in vitro* would not change the production of protein resulting from the expression of such genic transcripts. However, it is likely that there is no difference between groups in the protein expression too, once the synthesis of mineralized matrix did not differ significantly between groups.

The main factors that stimulate the differentiation of osteoprogenitor cells in the MSC have been studied. It is known that the proliferation and differentiation both *in vitro* as

well as *in vivo* of MSC in osteoblasts are under the command of several factors (Lindblad, 2001) such as genes, growth factors, mechanical stimulus and some hormones (Payushina *et al.*, 2006). These factors may act directly or indirectly in the various phases of osteogenic differentiation (Qu *et al.*, 1998; Hughes *et al.*, 2006) and little is known about the role of the PRL in each one of these steps and if such action is dose-dependent. Our results demonstrate that the action of prolactin *in vitro* on the MSC seems to be different from the action of this hormone in cultures of osteoblasts, despite of having been used concentrations similar to prolactin. The addition of prolactin in concentrations of 100 and 300ng increased cellularity, but did not promote significant changes of the parameters that allow us to evaluate the osteogenic differentiation of MSC, which suggests that the prolactin perhaps stimulates more the proliferative activity than the osteogenic differentiation of MSC. However, it cannot be ruled out that the reduction of osteogenic differentiation of the MSC is one of the mechanisms responsible for bone mass reduction observed in periods of hyperprolactinemia of gestation and lactation, since that i*n vivo*, the hyperprolactinemia may have an indirect effect on the osteogenic differentiation of MSC for reducing concentrations of estrogen, progesterone and gonadotropin hormones these that have direct action and positive about the osteogenic differentiation of MSC (Qu *et al.*, 1998; Dolinska *et al.,* 2010; Grachev *et al.*, 2015).

CONCLUSION

In conclusion, the addition of prolactin concentrations of 100ng/mL and 300ng/mL does not alter the osteogenic differentiation of ADSCs of female rats, despite increase of the cellularity of the culture.

ACKNOWLEDGEMENTS

This work was supported by grants from the Fundação de Amparo à Pesquisa de Minas Gerais (Fapemig) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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