Experimental Gerontology 113 (2018) 58–63

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Oxytocin effects on osteoblastic differentiation of Bone Marrow Mesenchymal Stem Cells from adult and aging female Wistar rats

Leandro Figueiredo Santos[a], Monique Patricio Singulani[a], Camila Tami Stringhetta-Garcia[a], Sandra Helena Penha Oliveira[a,b], Antonio Hernandes Chaves-Neto[a,b], Rita Cássia Menegati Dornelles[a,b,*]

[a] Programa de Pós-Graduação Multicêntrico em Ciências Fisiológicas, Unesp-SBFis, Brazil
[b] São Paulo State University (UNESP), School of Dentistry, Department of Basic Sciences, Araçatuba, Brazil

ARTICLE INFO

Section Editor: Werner Zwerschke

Keywords:
Aging
Bone marrow
Osteoblast
Oxytocin
Rat

ABSTRACT

Recently, it has been suggested that oxytocin (OT) might play a role in the control of bone remodeling and in bone health of young and adult females. The purpose of this study was to evaluate the effect of osteogenic medium (OM) plus OT (OM + OT; 100 nmol/L) on osteoblastic differentiation of bone marrow mesenchymal stem cells (BMMSCs) from cyclic adult (12 months old) and acyclic aging (24 months old) female Wistar rats. After 14 days, OM + OT increased the *oxytocin* and *oxytocin receptor* in the BMMSCs from animals of both age groups relative to OM controls. Alkaline phosphatase activity was higher in the OM + OT than OM group in BMMSCs from 24-month-old female rats. OM + OT improved osteogenic differentiation, observed by anticipated mineralization and increased gene expression of *bone morphogenetic protein 2*, *bone sialoprotein, osteopontin* and *osteocalcin* in both aged relative to OM controls. These findings suggest a role for OT as an adjuvant to induce osteoblastic differentiation of BMMSCs from aged female rat.

1. Introduction

The increase of life expectancy represents a major public health concern due to potential increase in vulnerability of aging women to diseases, such as osteoporosis (Cortet et al., 2011). In adult, bone structure is maintained by a highly dynamic and continuously renewed bone remodeling cycle, characterized by a sequence of activation-resorption performed by osteoclasts, followed by bone formation performed by osteoblasts. In postmenopausal osteoporosis, bone mass begins to decrease, along with estradiol serum levels and other hormones (Martin, 2007). During the aging process, bone turnover becomes affected by bone marrow mesenchymal stem cells (BMMSCs), specifically by precursors of osteoblast lineage cells, which fail to differentiate properly and disturb the activities of osteoblasts and osteoclasts (Kassem and Marie, 2011). Moreover, bone microenvironment is influenced by extrinsic and intrinsic factors that occur during life, implying that structural abnormalities associated with osteoporotic bone may be a consequence of inadequacies in bone cell differentiation (Asumda and Chase, 2011).

In order to understand the cellular events necessary for bone formation, studies have analyzed central and peripheral substances with potential to cause osteogenic differentiation. Previous studies have investigated the action of oxytocin (OT) in bone cells. This hormone is synthesized in the hypothalamus, released into circulation by the neurohypophysis, and has a primary function in milk ejection and uterine contraction in a reproductive age (Blanks and Thornton, 2003; Breton et al., 2001). A beneficial action of this neurohormone related to bone metabolism was reported for osteoblastic cells of young animals (Petersson et al., 2002). Studies performed with 6- and 12-month-old ovariectomized rats treated with peripheral OT exhibited positive effects in the activity of osteoblasts (Colaianni et al., 2011; Cuscito et al., 2011; Elabd et al., 2008; Tamma et al., 2009). Another work using the same study model showed beneficial effects on the bone tissue after administration of OT in 6-month-old animals (Beranger et al., 2014). Considering that the female aging process is a results of neuroendocrine signaling changes (Ferreira et al., 2015), global hormonal variation, such as estrogen deficiency (Nicola et al., 2016; Stringhetta-Garcia et al., 2016), as well as BMMSCs loss of capacity to differentiate into osteoblasts (Singulani et al., 2017), the purpose of this study was to evaluate the influence of OT in osteogenic differentiation of BMMSCs

* Corresponding author at: Department of Basic Sciences, Araçatuba Dental School, Univ. Estadual Paulista - UNESP, Rodovia Marechal Rondon, km 527, 16018-805 Araçatuba, SP, Brazil.
E-mail address: rita.dornelles@unesp.br (R.C.M. Dornelles).

https://doi.org/10.1016/j.exger.2018.09.023
Received 12 April 2018; Received in revised form 21 September 2018; Accepted 25 September 2018
Available online 27 September 2018

from cyclic adult (12 months old) and acyclic aging (24 months old) female Wistar rats.

2. Materials and methods

2.1. Animals

Multiparous female rats (*Rattus norvegicus albinus*) at ages 12 and 24 months were housed in standard cages with ad libitum access to food and water in a temperature-controlled environment with a 12 h light/dark cycle. Estrous cycle was checked through vaginal smears taken daily to confirm regularity in the cycle of adult rats, and irregularity in the cycle of aged females, characterizing the estropause period (Bestetti et al., 1991). Selected cyclic (12-month-old group) and acyclic (24-month-old group) animals were used for BMMSCs isolation according to protocol described previously (Wang et al., 2012). Animal procedures were approved by Ethics Committee on Animal Use São Paulo State University (Unesp), School of Dentistry, Araçatuba (Protocol number 00981/2012).

2.2. Isolation and culture of BMMSCs

After euthanasia by anesthetic overdose, femurs were surgically removed, cleaned of the adjacent tissue, and BMMSCs were harvested by flushing out the bone marrow with proliferative culture medium (MEM - minimum essential medium; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 4 mmol/L L-glutamine, 10 μg/mL gentamicin, 0.25 μg/mL amphotericin B, 100 units/mL penicillin G, and 100 μg/mL streptomycin (Gibco/Life Technologies, Paisley, UK). After rapid centrifugation, the cells pellet was resuspended in culture medium, and then seeded in 75-cm^2 culture flasks with proliferative culture medium in a humidified atmosphere of 5% CO_2 at 37 °C. Non-adherent cells were removed after 48 h by washing with PBS. The medium was changed subsequently every 3 days. For all experiments, after reaching 80–90% confluence, BMMSCs were recovered using 0.25% trypsin-EDTA (Gibco/Life Technologies, Paisley, UK) and plated at a density of 3000 cell/cm^2 surface area. After reaching 70–80% confluence (first passage), BMMSCs from cyclic and acyclic rats were divided into two groups each: osteogenic medium (OM) [5.5 mmol/L glucose, 50 μg/mL ascorbic acid, 10 mmol/L β-glycerophosphate, 10 nmol/L dexamethasone]; OM plus 100 nmol/L OT (OM + OT) (Copland et al., 1999; Tamma et al., 2009). All medium culture was changed subsequently every three days until analysis.

2.3. Viability assay

MTT (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma Aldrich™) method (Mosmann, 1983) was performed on experimental days 3, 7 and 14. MTT was added to each sample to allow the formation of MTT formazan. After 4 h of incubation, optical density (OD) was measured at 570 nm by a spectrophotometric microplate reader (Molecular Devices', CA, USA). The results were expressed in OD.

2.4. Alkaline phosphatase specific activity

ALP activity was measured on experimental days 3, 7 and 14 using a specific commercial kit (Labtest Diagnóstica, Brazil) based on the method of Roy (Roy, 1970). The specific activity of ALP (U/mg total protein) was calculated from the measured standard tube and normalized by total protein content, that was determined by the method of Lowry and contributors (Lowry et al., 1951).

2.5. Alizarin Red S staining assay for calcium phosphate deposition

Alizarin Red mineralization assay (Gregory et al., 2004) was performed on experimental days 14 and 17. Cells were fixed with paraformaldehyde (Merck) at 10%, washed with distilled water, and stained with 20 mg/mL Alizarin Red S (Sigma Aldrich™) (pH 4.2). After drying at room temperature, the plates were digitalized using a high-resolution scanner, staining was removed with acetic acid solution (Sigma Aldrich™) at 10% and samples were spectrophotometrically measured at 405 nm using a microplate reader (Molecular Devices', CA, USA). The results were expressed in OD.

2.6. Relative quantification in real-time PCR

On experimental day 14, total RNA was isolated from cells by using Trizol (Invitrogen, Life Technologies, NY, USA) and other conventional reagents like chloroform (MERCK), isopropanol (MERCK), ethanol (MERCK) and DNase I. RNA was then reverse transcribed to complementary DNA (cDNA) using *SuperScript*™ II Reverse Transcriptase (Invitrogen). Expression of target genes was determined by real-time PCR using gene-specific TaqMan® probes with TaqMan® Universal Master Mix II (Applied Biosystems) in a StepOne plus™ Real-Time PCR System (Applied Biosystems Life Technologies) device.

The following primers and probes labeled with FAM fluorophore (Applied Biosystems Life Technologies) were used: *Oxytocin* (*Oxt* - Rn00564446_g1); *Oxytocin receptor* (*Oxtr* - Rn00563503_m1); *Osterix* (*Osx* - Rn02769744_s1); *Bone morphogenetic protein 2* (*Bmp2*- Rn.90931); *Collagen type I alpha 1 chain* (*Col1a1-Rn01463848_m1*); *Osteocalcin* (*Ocn* - Rn00566386_g1); *Bone sialoprotein* (*Bsp* - Rn00561414_m1) and *Osteopontin* (*Opn* - Rn00563571_m1). Expression levels of target genes were determined by relative quantification (RQ), using the equation $RQ = 2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Changes in gene expression were calculated using relative quantitation of a target gene against endogenous *β-actin* (*Actb* - Rn00667869_m1). Samples from BMMSCs of the OM group at each age were used as a calibrator.

2.7. Statistical analysis

Data are expressed as mean ± standard errors of the mean (SEM) for at least three independent experiments. Parametric data were analyzed by determined by one-way ANOVA followed by the Tukey post-test for the MTT, ALP and Alizarin Red assays. Real time PCR experiments were assessed using the two-tailed unpaired Student's t-test. Significance level was set at 5% ($p < 0.05$) for all comparisons. All analyses were performed using the statistical software GraphPad Prism, version 6.0 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Oxytocin increases MTT reduction of BMMSCs

MTT reduction into formazan (Mosmann, 1983) was performed on experimental days 3, 7 and 14 to determine whether OT + OM influences the viability assay of BMMSCs obtained from adult and aged rats (Fig. 1A and B). On day 3 of osteogenic induction, the OM + OT groups of both ages showed a higher MTT activity when compared to the OM groups ($p < 0.001$/12 months, $p < 0.05$/24 months; Fig. 1A and B). In cells of 24-month-old animals, this increase was maintained on day 7 ($p < 0.05$; Fig. 1B). However, cells from the 12-month-old adult rats OM + OT group showed a lower capacity for MTT reduction on day 14 ($p < 0.0001$; Fig. 1A).

3.2. ALP activity in oxytocin-treated aged osteogenic cells

Analysis of ALP activity is used as a marker of early stages of mineralization (Lian and Stein, 1995). ALP activity in BMMSCs from 12-month-old animals was lower in the OM group when compared days 3 with 14 ($p < 0.01$; Fig. 1C). In cells from 24-month-old rats, ALP activity was significantly increased in the OM + OT group compared to