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ORIGINAL ARTICLE

Jabuticaba peel extract modulates adipocyte and osteoblast differentiation of MSCs from healthy and osteoporotic rats

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Abstract

Introduction The jabuticaba peel extract (JPE) contains bioactive compounds that regulate fat metabolism. Because the negative correlation between fat accumulation and bone formation in bone marrow, we hypothesized that JPE inhibits adipocyte as well as favors osteoblast differentiation of mesenchymal stromal cells (MSCs) under healthy and osteoporotic conditions, a disease that display an imbalance between adipocyte and osteoblast differentiation resulting in reduced bone mass.

Material and methods To test these hypotheses, bone marrow MSCs were harvested from healthy and osteoporotic rats and cultured in adipogenic and osteogenic media with three concentrations of JPE, 0.25, 5 and 10 µg/ml, and vehicle (control). After selecting the most efficient concentrations of JPE, we used them to evaluate adipocyte and osteoblast differentiation of MSCs from both sources.

Results We observed that, in general, JPE inhibited adipocyte differentiation of MSCs with more pronounced effects in cells from healthy than osteoporotic rats. In addition, JPE increased osteoblast differentiation, exhibiting a slightly higher osteogenic potential on MSCs from osteoporotic compared to healthy condition.

Conclusion Our results demonstrated that JPE drives MSCs to inhibit adipocyte differentiation and toward osteoblast differentiation under healthy and osteoporotic conditions. These findings pave the way for further translational studies to investigate the therapeutic possibilities of JPE in both prevention and treatment of osteoporosis.

Keywords Adipocyte · Jabuticaba · Mesenchymal stromal cell · Osteoblast · Osteoporosis

Introduction

Products of natural origin have been increasingly used as a viable alternative to treat several diseases, including those that cause damage to bone tissue [1–3]. The raw materials of these products are easily found in nature, such as fruits, vegetables, herbs and nuts, the peels, roots, leaves and stems of which are used due to their beneficial properties [4, 5]. The main advantages that make natural products a safe and affordable alternative compared to synthetic drugs currently available are natural growth, high availability, reduced production costs, minimal side effects and low toxicity [6–8].

Jabuticaba (*Myrciaria jaboticaba* (Vell.) Berg.) is a Brazilian fruit measuring 3–4 cm in diameter similar to blueberry and its purple peel concentrates most of its bioactive compounds, such as polyphenols, flavonoids and anthocyanins [9, 10]. These compounds are directly related to health benefits and several studies have shown that jabuticaba consumption may prevent weight gain, dyslipidemia and hyperglycemia, as well as control cholesterol levels [11–14]. In addition to these metabolic effects, the jabuticaba peel extract (JPE) may also prevent liver steatosis in high-fat-fed aging mice [15]. To maintain the properties of the bioactive compounds and have precise control of the ingested doses, the JPE was developed as a processed product of easy

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administration, becoming a promising candidate as a therapeutic agent for several diseases [10, 15]. Despite the open therapeutic possibilities of JPE, up to now, its effects on bone cells from normal and unbalanced conditions, such as osteoporosis, have not yet been addressed.

Osteoporosis is the most common metabolic bone disorder characterized by low bone mineral density due to an imbalance in bone remodeling, resulting in more porous bones, with fragile microarchitecture and more susceptibility to fractures [16, 17]. The main cells involved in the pathophysiology of osteoporosis are osteoclasts derived from hematopoietic stem cells and osteoblasts derived from bone marrow mesenchymal stromal cells (MSCs) [18, 19]. The MSCs are involved in the maintenance of bone homeostasis and characterized as multipotent cells capable of differentiating into various cell types, including adipocytes and osteoblasts [20]. In osteoporosis, as well as in aging, there is an imbalance in the differentiation of bone marrow MSCs in favor of adipocytes at the expense of osteoblasts, which results in increased fat accumulation and decreased bone formation in the bone marrow microenvironment [21–23]. Because of the adipocyte accumulation, several adipokines are secreted in substantial amounts, which not only inhibit bone formation, but also stimulate bone resorption, thus contributing to bone fragility [24].

To keep the balance of bone mass, several synthetic drugs have been used to treat osteoporosis, either to inhibit bone resorption or to induce bone formation, and it has been shown that some of them may act on both sides [25–27]. However, despite the benefits, the use of currently approved treatments for osteoporosis is associated with side effects such as atypical femoral fracture, osteonecrosis of the jaw, dizziness, fainting, risk of thromboembolism, muscle pain and skin problems [28, 29]. These adverse effects could be reduced or even eliminated by using natural products to treat osteoporosis. In addition to stimulating bone cells in vitro, some of these natural products favor in vivo bone formation under osteoporotic conditions [30–34].

Despite the ability of JPE to affect the lipid metabolism as described above, to date, it is not known if JPE can modulate MSCs commitment and differentiation under healthy and osteoporotic conditions, where the bone marrow adiposity is increased. Thus, due the anti-adipogenic properties of JPE and the inverse correlation between adipose accumulation and bone formation in the bone marrow, we hypothesized that JPE can reduce adipocyte differentiation as well as stimulate osteoblast differentiation of MSCs. Therefore, the aim of this study was to evaluate the adipogenic and osteogenic effects of JPE using MSCs from healthy and osteoporotic rats.

Materials and methods

Animals

All animal experiments were approved by the Committee of Ethics in Animal Research of the School of Dentistry of Ribeirão Preto, University of São Paulo (CEUA # 2018.1.30.58.8) and followed all institutional, national and international guidelines for the care and use of animals. We used a total of 16 animals: 12 6-month-old female Wistar rats of either ovariectomy ($n=8$) or SHAM ($n=4$) surgical procedures and 4 4-week-old healthy male Wistar rats ($n=4$).

Surgical procedure to induce osteoporosis

Postmenopausal osteoporosis was induced through bilateral ovariectomy (OVX), as previously described [35]. Briefly, 6-month-old female Wistar rats ($n=8$) were anesthetized and the surgery site was cleaned. Then, a bilateral abdominal incision was performed to expose and remove the ovaries, and the skin was sutured with nylon suture threads 4.0 (Ethicon, Sao Paulo, SP, Brazil). The control group (SHAM) was composed of 6-month-old female Wistar rats ($n=4$) submitted to the same surgical intervention, excepting the removal of ovaries, to reproduce the effects of the surgical stress. As postoperative care, single doses of antibiotics and analgesics were administered.

Ninety days after surgical procedures, the microtomographic analysis (μCT) was carried out to verify the osteoporotic condition. The OVX and SHAM rats ($n=4$ for each group) were euthanatized with an overdose of CO_2, and the femurs were removed and stored in 10% formalin pH 7.0 (Merck, HE, Germany). The samples were scanned in high-resolution SkyScan 1172 microtomography (Bruker, Kontich, Belgium) and the images were obtained at 9.8 pixel size, 60 kV, and 165 μA. The NRecon software (version 1.6.10.4, Bruker) was used for the reconstructions with the following parameters: smoothing set at 2, ring artifact correction set at 6, and beam hardening correction set at 20%. The reconstructions were analyzed by the CTAn software (version 1.15.4.0, Bruker) to evaluate the morphometric parameters, bone mineral density (BMD), bone volume/total volume (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th).

Cell isolation

The MSCs were obtained from bone marrow of 4-week-old healthy male Wistar rats (MSCs) and from ovariectomized rats (OVX-MSCs) 5 months post-surgery which mimics postmenopausal osteoporosis ($n=4$ for each group).

Briefly, the animals were euthanized as described above, both femurs were removed, the cells were flushed from them and cultured until subconfluence in growth medium, composed of minimum essential medium alpha modification (α-MEM, Gibco-Life Technologies, Grand Island, NY, USA), 10% fetal bovine serum (Gibco-Life Technologies), 50 µg/ml gentamicin (Gibco-Life Technologies) and 0.3 µg/ml of fungizone (Gibco-Life Technologies). Both MSCs and OVX-MSCs were kept at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air, and the culture medium was changed every 48 h.

Synthesis and bioactive compounds content of the JPE

The jabuticabas were kindly donated by Indústria e Comércio Lagoa Branca Ltda (Casa Branca, SP, Brazil). They were washed and manually peeled, the peel was dried in a stove with air circulation (Marconi, Piracicaba, SP, Brazil) at 40 °C for 72 h and the dried peels were ground with an electric mill (MA 630/1, Marconi) and sifted (mash 20).

The JPE was obtained by high-intensity ultrasound-assisted extraction using a 13-mm ultrasonic probe diameter at 19 kHz (Unique, Desruptor, Indaiatuba, SP, Brazil) for 3 min. The probe contact height with the extract was kept at 40 mm and an ice bath was used to prevent the extract from overheating. The dried jabuticaba peel (1 g) and 25 ml of solvent extraction (50% ethanol absolute in ultrapure water, w/w) were used at a nominal power of 320 W. The extraction was made in triplicate and the solvent was removed with vacuum. The dried extract was reconstituted in the same volume of ultrapure water and freeze-dried and stored at − 20 °C until use.

The freeze-dried JPE was reconstituted in ultrapure water at a concentration of 6.33 mg/ml for the quantitative analyses of polyphenolic and anthocyanin contents. The total polyphenolic content measured by reducer capacity was determined by the Folin–Ciocalteu method adapted from a previous description [36]. The absorbance was measured at 725 nm in a plate reader µQuant (Bio-Tek Instruments Inc., Winooski, VT, USA), gallic acid was used in a standard curve and the data were expressed in gallic acid equivalent (mg GAE/L). The monomeric anthocyanins were quantified according to the pH-differential method [37] and the data were expressed in mg/L.

Selection of JPE concentration

To select the more efficient concentrations of JPE, three different doses were added to the cultures of both MSCs and OVX-MSCs. Briefly, the cells cultured in growth medium until subconfluence, as described above, were then cultured in 24-well polystyrene plates (Corning Incorporated, Corning, NY, USA) at a density of 2×10^4 cells/well either in adipogenic medium, composed of growth medium supplemented with 10^{-6} M dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 0.5 µM 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 10 µg/ml insulin (Sigma-Aldrich), and 0.1 M indomethacin (Sigma-Aldrich), or in osteogenic medium, composed of growth medium supplemented with 50 µg/ml ascorbic acid (Gibco-Life Technologies), 7 mM β-glycerophosphate (Sigma-Aldrich), and 10^{-7} M dexamethasone (Sigma-Aldrich). Under both adipogenic and osteogenic conditions, cells were treated with 0.25, 5 and 10 µg/ml of JPE during the entire period of culture and cells grown in the presence of the vehicle (distilled water) were used as control. Then, the adipogenesis was evaluated by lipid accumulation and osteogenesis by alkaline phosphatase (ALP) activity as described below.

Lipid accumulation

On day 21, the lipid accumulation was detected by Oil red O staining of both MSCs and OVX-MSCs cultured in adipogenic medium with the different concentrations of JPE ($n = 5$), as described above. Briefly, the cells were fixed in 10% formalin for 2 h at room temperature, washed with isopropanol 60% (Merck) and stained with 0.3% Oil red O (Sigma-Aldrich) for 10 min. Then, the images of lipid drops were acquired using a light microscopy Axiovert 25 (Zeiss, Germany) coupled to a high-resolution camera (Canon EOS Digital Rebel, Canon, Japan).

ALP activity

On day 10, the ALP activity was detected by Fast Red staining in both MSCs and OVX-MSCs cultured in osteogenic medium with the different concentrations of JPE ($n = 5$), as described above. Briefly, 0.8 ml of a solution of 1.8 mM Fast Red-TR 1,5-naphthalenedisulfonate salt (Sigma-Aldrich) and 0.9 mM naphthol AS-MX phosphate (Sigma-Aldrich) were added to the samples, which were kept at room temperature for 30 min. Then, the solution was removed, and the plates were dried overnight at room temperature. The images of the samples were acquired with a high-resolution camera (Canon EOS Digital Rebel).

Effect of JPE on adipocyte differentiation of MSCs and OVX-MSCs

To evaluate the effect of JPE on adipocyte differentiation, both MSCs and OVX-MSCs were cultured in 24-well polystyrene plates (Corning Incorporated) in adipogenic medium, as described above, containing JPE at the previously selected concentration for each cell type. The cells were kept at 37 °C in a humidified atmosphere containing

5% CO_2 and 95% air for up 21 days, and the medium was changed every 48 h. Then, the gene expression of some adipocyte markers as well as lipid accumulation was evaluated.

On day 5, real-time PCR was carried out to evaluate the gene expression of adiponectin (*Adipoq*), resistin (*Retn*), adipocyte protein 2 (*aP2*), and peroxisome proliferator-activated receptor gamma (*Pparγ*). Briefly, the RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), and the concentration and purity were analyzed following the manufacturer's instructions. The complementary DNA (cDNA) was synthesized using an equal amount of RNA (1 µg) through a reverse transcription reaction (Kit High Capacity, Invitrogen). Real-time PCR was carried out in a Step One Plus Real-Time PCR (Gibco-Life Technologies) with Taqman Master Mix and probes for adipocyte markers. The beta-actin (*β-actin*) and eukaryotic translation initiation factor 2B (*Eif2b)* were used as housekeeping genes and the relative gene expression ($n=3$) was calculated by the $2^{-\Delta\Delta CT}$ method, and *β-actin* was selected for normalization based on its cycle threshold value. The TaqMan probes used in this study for real-time PCR are presented in Table 1.

The lipid accumulation was evaluated on day 21, as described above. Additionally, after acquiring the images, the incorporated Oil red O was extracted by incubation with 100% isopropanol for 10 min under shaking at room temperature. After appropriate dilution, the lipid accumulation was measured using a colorimetric method, the samples were read at 500 nm in a plate reader µQuant (Bio-Tek Instruments Inc.), and the data ($n=5$) were expressed as absorbance.

Effect of JPE on osteoblast differentiation of MSCs and OVX-MSCs

To evaluate the effect of JPE on osteoblast differentiation, both MSCs and OVX-MSCs were cultured in 24-well polystyrene plates (Corning Incorporated) in osteogenic medium, as described above, containing JPE at the previously selected concentration for each cell type. The cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air for up 21 days, and the medium was changed every 48 h. Then, the gene expression of some osteoblast markers, ALP activity and extracellular matrix mineralization was evaluated.

On day 5, real-time PCR was carried out to evaluate the gene expression of runt-related transcription factor 2 (*Runx2*), osterix (*Sp7*), osteocalcin (*Oc*) and osteopontin (*Opn*). The *β-actin* and *Eif2b* were used as housekeeping genes, the relative gene expression ($n=3$) was calculated by the $2^{-\Delta\Delta CT}$ method, and *β-actin* was selected for normalization based on its cycle threshold value. The TaqMan probes used in this study for real-time PCR are presented in Table 1.

The ALP activity was evaluated on day 10, as described above and after acquiring the images, the quantification was performed by counting the pixels of each sample, using ImageJ software, version 1.5i (National Institutes of Health, Bethesda, MD, USA), and the data ($n=5$) were expressed as a percentage of area.

On day 21, the extracellular matrix mineralization was detected by Alizarin red staining. Briefly, the samples were fixed in 10% formalin for 2 h at room temperature, dehydrated, stained with Alizarin red S (Sigma-Aldrich), pH 4.2, for 10 min and dried at room temperature. The images of the samples were acquired with a high-resolution camera (Canon EOS Digital Rebel). Then, to detect calcium content, 280 µl of 10% acetic acid was added to each well and the plate was incubated at room temperature for 30 min under shaking. This solution was vortexed for 1 min, heated at 85 °C for 10 min, and transferred to ice for 5 min. The slurry was centrifuged at 13,000 *g* for 15 min and 100 µl of the supernatant was mixed with 40 µl of 10% ammonium hydroxide. This solution was read at 405 nm in a plate reader µQuant (Bio-Tek Instruments Inc.), and the data ($n=5$) were expressed as absorbance.

Table 1 TaqMan probes for real-time PCR

Gene	Gene name	Identification
Adipoq	Adiponectin	Rn00595250_m1
Retn	Resistin	Rn00595224_m1
aP2	Fatty acid binding protein 4 (adipocyte protein 2)	Rn04219585_m1
Ppary	Peroxisome proliferator-activated receptor gamma	Rn00440945_m1
Runx2	Runt-related transcription factor 2	Rn01512298_m1
Sp7	Sp7 transcriptor factor (osterix)	Rn02769744_s1
Oc	Bone gamma-carboxyglutamate protein (*Bglap*/osteocalcin)	Rn00566386_g1
Opn	Secreted phosphoprotein 1(Ssp1/osteopontin)	Rn01449972_m1
β-actin	Beta-actin	Rn00667869_m1
Eif2b	Eukaryotic translation initiation factor 2B	Rn00672503_m1

Statistical analysis

Numerical data were expressed as mean ± standard deviation. The comparisons were done between SHAM and OVX rat femurs, to verify the effect of osteoporosis on bone morphometric parameters, as well as between the absence and presence of JPE on both adipocyte and osteoblast differentiation of MSCs and OVX-MSCs. As the data followed the normal distribution, they were compared using the Student's *t* test using SigmaPlot Software (Systat, Erkrath, Germany). The level of significance was set at $p \leq 0.05$.

Results

Effect of osteoporosis on bone morphometric parameters

The effect of osteoporosis on bone tissue was detected by µCT analysis and the three-dimensional reconstructed images revealed that the distal epiphysis of femurs from SHAM rats (Fig. 1a) exhibited higher trabecular density compared to those from OVX rats (Fig. 1b). Moreover, the bone morphometric parameters, BMD (Fig. 1c), BV/TV (Fig. 1d) and Tb.N (Fig. 1e), were lower in OVX rats compared to SHAM rats ($p = 0.026$, $p = 0.029$ and $p = 0.030$, respectively), and Tb.Sp (Fig. 1f) was higher in OVX rats compared to SHAM rats (p = 0.014), while Tb.Th (Fig. 1g) was not different between SHAM and OVX rats ($p = 0.474$).

Bioactive compound content of the JPE

The JPE presented 7362 ± 53 mg GAE/L of the total polyphenolic content and 710 ± 4 mg/L of the monomeric anthocyanins. These values were highest when compared to studies that used conventional methods for extraction [10, 13].

Selection of JPE concentration

As expected, the adipogenic and osteogenic potentials were different between MSCs and OVX-MSCs. In all situations, regardless of the presence of different concentrations of JPE, the lipid accumulation was higher in OVX-MSCs than in MSCs (Fig. 2a), while ALP activity was higher in MSCs than in OVX-MSCs (Fig. 2b). When healthy and osteoporotic conditions were separately evaluated, the lipid accumulation was lower in the presence of 0.25 µg/ml of JPE in both cultures of MSCs and OVX-MSCs (Fig. 2a). The JPE at concentrations of 0.25 µg/ml in MSCs and 10 µg/ml in OVX-MSCs induced a higher ALP activity when compared to the control and all other JPE concentrations (Fig. 2b). Thus, based on these results, we selected the concentration of 0.25 µg/ml to evaluate the effect of JPE on both adipocyte and osteoblast differentiation of MSCs. Furthermore, the concentrations of 0.25 µg/ml and 10 µg/ml were chosen

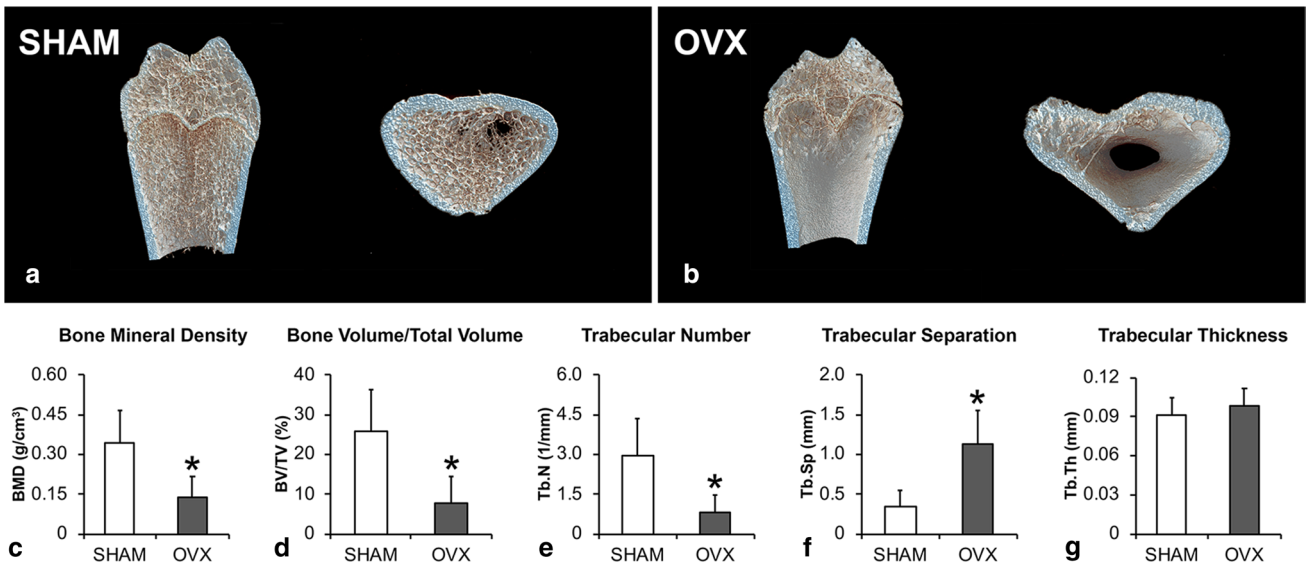

Fig. 1 Effect of osteoporosis on bone morphometric parameters. Three-dimensional reconstructed microtomographic (µCT) images (**a**, **b**) and morphometric parameters (**c**–**g**) of distal epiphysis of femurs from healthy (SHAM) and osteoporotic (OVX) rats, 90 days after the surgical procedure. The SHAM femurs (**a**) exhibited higher trabecular density than OVX (**b**). The data of bone mineral density (**c**, BMD), bone volume/total volume (**d**, BV/TV), trabecular number (**e**, Tb.N), trabecular separation (**f**, Tb.Sp), and trabecular thickness (**g**, Tb.Th) are presented as mean ± standard deviation ($n = 4$). Asterisks (*) indicate statistically significant difference between SHAM and OVX rats (Student's *t* test, $p \leq 0.05$)

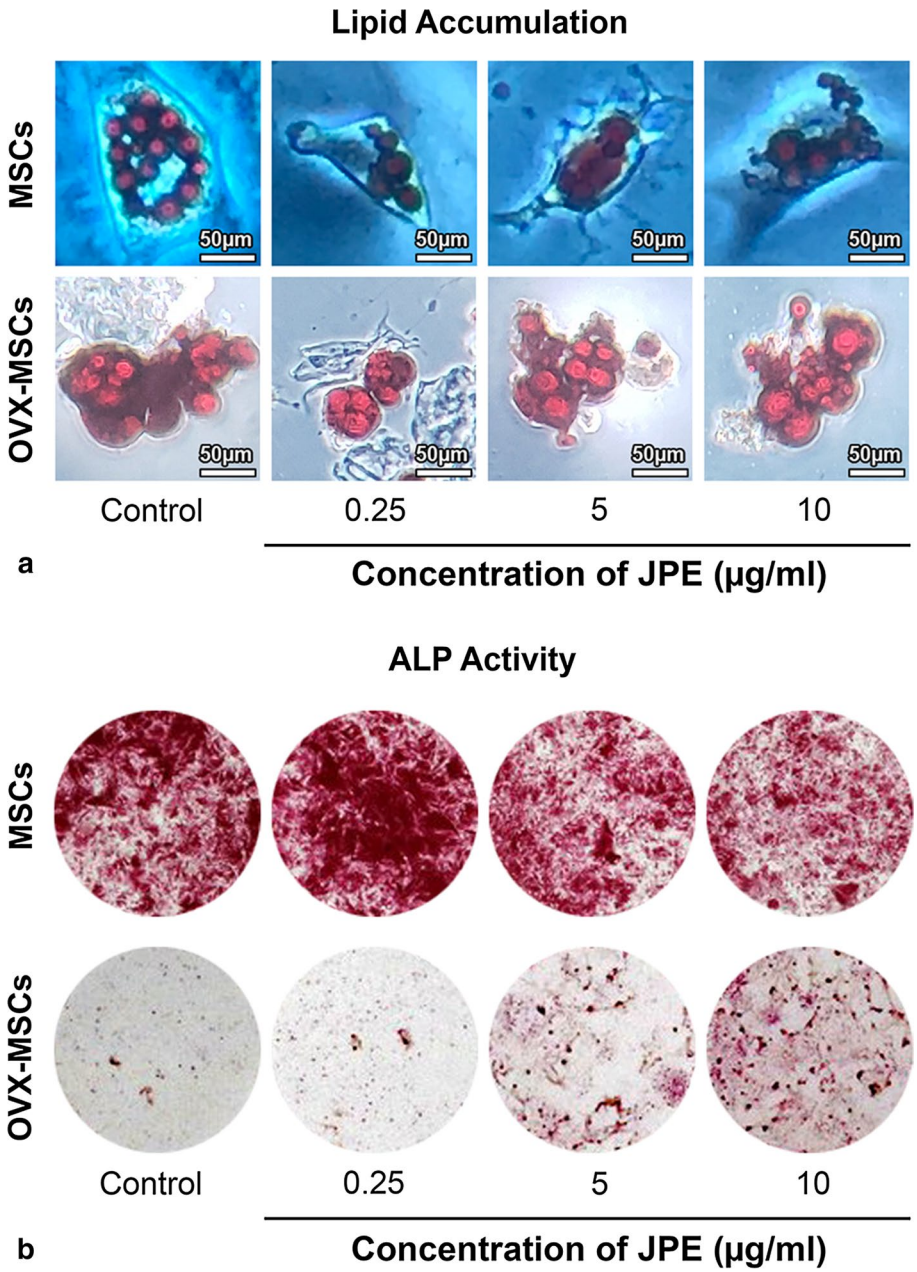

Fig. 2 Selection of JPE concentration. Lipid accumulation (**a**) on day 21 and alkaline phosphatase (ALP) activity (**b**) on day 10 of bone marrow-derived mesenchymal stromal cells from healthy (MSCs) and osteoporotic (OVX-MSCs) rats cultured in adipogenic medium (**a**) or osteogenic medium (**b**) without (Control) or with 0.25 μg/ml, 5 μg/ml and 10 μg/ml of JPE

to evaluate the effect of JPE on adipocyte and osteoblast differentiation of OVX-MSCs, respectively.

Effect of JPE on adipocyte differentiation of MSCs

To detail the effect of JPE at a concentration of 0.25 μg/ml on adipocyte differentiation of MSCs cultured under adipogenic condition, at both genotype and phenotype levels, we evaluated the gene expression of key adipocyte markers on day 5 and lipid accumulation on day 21. We observed that the gene expression of *Adipoq* (Fig. 3a, $p = 0.001$), *Retn* (Fig. 3b, $p = 0.001$) and *aP2* (Fig. 3c, $p = 0.005$) was downregulated, while *Pparγ* (Fig. 3d, $p = 0.081$) was not affected by JPE. Additionally, JPE decreased lipid accumulation (Fig. 3e, $p = 0.009$).

Effect of JPE on osteoblast differentiation of MSCs

To investigate the effect of JPE at a concentration of 0.25 μg/ml on osteoblast differentiation of MSCs cultured under osteogenic condition, at both genotype and phenotype levels, we evaluated the gene expression of key osteoblast markers on day 5, ALP activity on day 10 and extracellular matrix mineralization on day 21. Despite JPE did not affect the gene expression of *Runx2* (Fig. 4a, $p = 0.870$), *Sp7* (Fig. 4b, $p = 0.129$), *Oc* (Fig. 4c, $p = 0.079$) and *Opn*

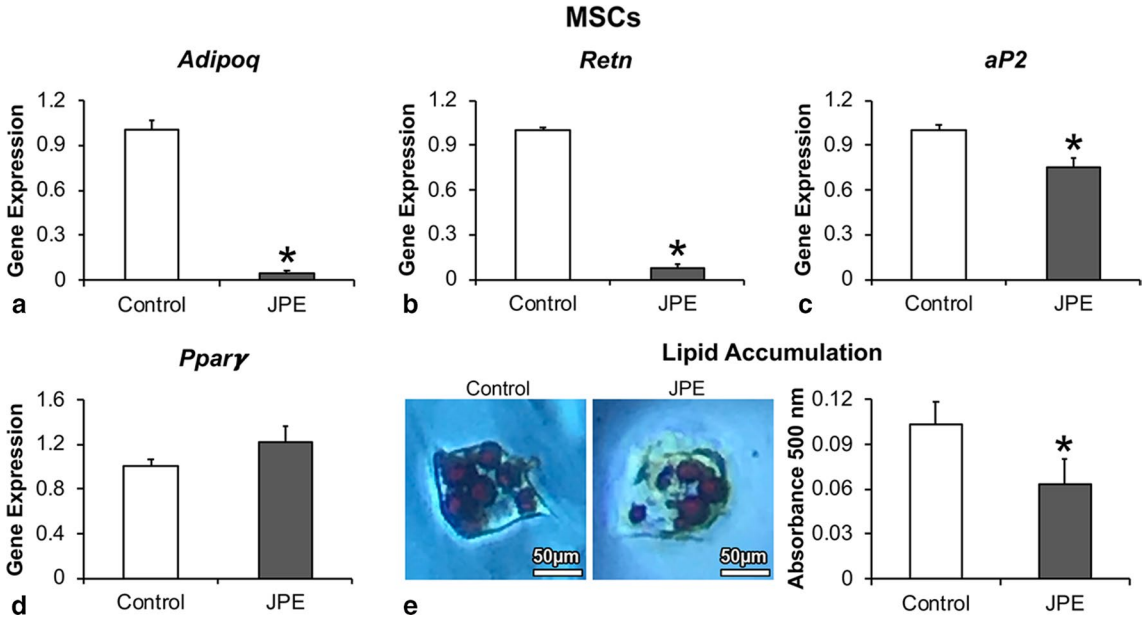

Fig. 3 Effect of JPE on adipocyte differentiation of bone marrow-derived mesenchymal stromal cells from healthy rats (MSCs). Gene expression of the adipocyte markers adiponectin (*Adipoq*, **a**), resistin (*Retn*, **b**), adipocyte protein 2 (*aP2*, **c**), and peroxisome proliferator-activated receptor gamma (*Pparγ*, **d**) on day 5, and quantification of lipid accumulation on day 21 (**e**) of MSCs cultured in adipogenic medium without (Control) or with 0.25 μg/ml of JPE. The data of gene expression ($n=3$) and lipid accumulation ($n=5$) are presented as mean ± standard deviation. Asterisks (*) indicate statistically significant difference between Control and JPE (Student's *t* test, $p \leq 0.05$)

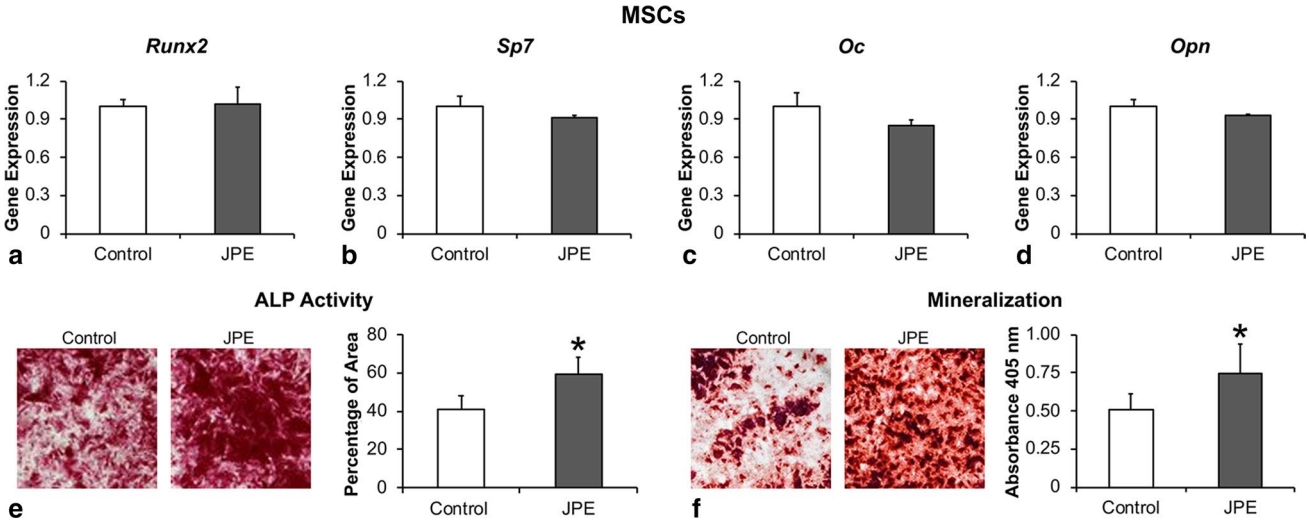

Fig. 4 Effect of JPE on osteoblast differentiation of bone marrow-derived mesenchymal stromal cells from healthy rats (MSCs). Gene expression of the osteoblast markers runt-related transcription factor 2 (*Runx2*, **a**), osterix (*Sp7*, **b**), osteocalcin (*Oc*, **c**) and osteopontin (*Opn*, **d**) on day 5, and quantification of alkaline phosphatase (ALP) activity on day 10 (**e**) and extracellular matrix mineralization on day 21 (**f**) of MSCs cultured in osteogenic medium without (Control) or with 0.25 μg/ml of JPE. The data of gene expression ($n=3$), ALP activity ($n=5$) and extracellular matrix mineralization ($n=5$) are presented as mean ± standard deviation. Asterisks (*) indicate statistically significant difference between Control and JPE (Student's *t* test, $p \leq 0.05$)

(Fig. 4d, $p=0.089$), it increased both ALP activity (Fig. 4e, $p=0.006$) and extracellular matrix mineralization (Fig. 4f, $p=0.032$).

Effect of JPE on adipocyte differentiation of OVX-MSCs

We evaluated the same parameters described for MSCs to verify the effect of JPE at a concentration of 0.25 µg/ml on adipocyte differentiation of OVX-MSCs cultured under adipogenic condition. The JPE did not affect the gene expression of *Adipoq* (Fig. 5a, $p=0.676$), *Retn* (Fig. 5b, $p=0.111$) and *Pparγ* (Fig. 5d, $p=0.745$), and reduced the gene expression of *aP2* (Fig. 5c, $p=0.004$) as well as the lipid accumulation (Fig. 5e, $p=0.009$).

Effect of JPE on osteoblast differentiation of OVX-MSCs

As the higher osteogenic potential of JPE in OVX-MSCs was observed with 10 µg/ml, we evaluated its effect on osteoblast differentiation using the same parameters described for MSCs. The gene expression of *Runx2* (Fig. 6a, $p=0.766$) was not affected, while that of *Sp7* (Fig. 6b, $p=0.011$), *Oc* (Fig. 6c, $p=0.013$) and *Opn* (Fig. 6d, $p=0.001$) was upregulated by JPE. In addition, JPE increased ALP activity (Fig. 6e, $p=0.001$) as well as the extracellular matrix mineralization (Fig. 6f, $p=0.031$).

Discussion

Considering the critical role of MSCs in the maintenance of bone homeostasis and the ability of JPE to regulate lipid metabolism, here, we evaluated the effect of JPE on adipocyte and osteoblast differentiation of MSCs from healthy and osteoporotic rats. We have shown that JPE affects MSCs differentiation, favoring osteoblast and inhibiting adipocyte differentiation under both healthy and osteoporotic conditions, which makes this natural compound a promising candidate for further studies of therapies to prevent and treat osteoporosis.

The postmenopausal osteoporosis increases bone resorption and accelerates the cancellous bone loss in women with estrogen deficiency; therefore, an animal model to mimic this type of osteoporosis should have these same characteristics [38]. To achieve this, the surgical procedure of ovariectomy has been used over the last 30 years and has proven to be the most popular and efficient method to induce osteoporosis in rats [35, 38–41]. Here, we were able to reproduce the deleterious effects of the lack of estrogen on bone tissue induced by ovariectomy and consequently it makes

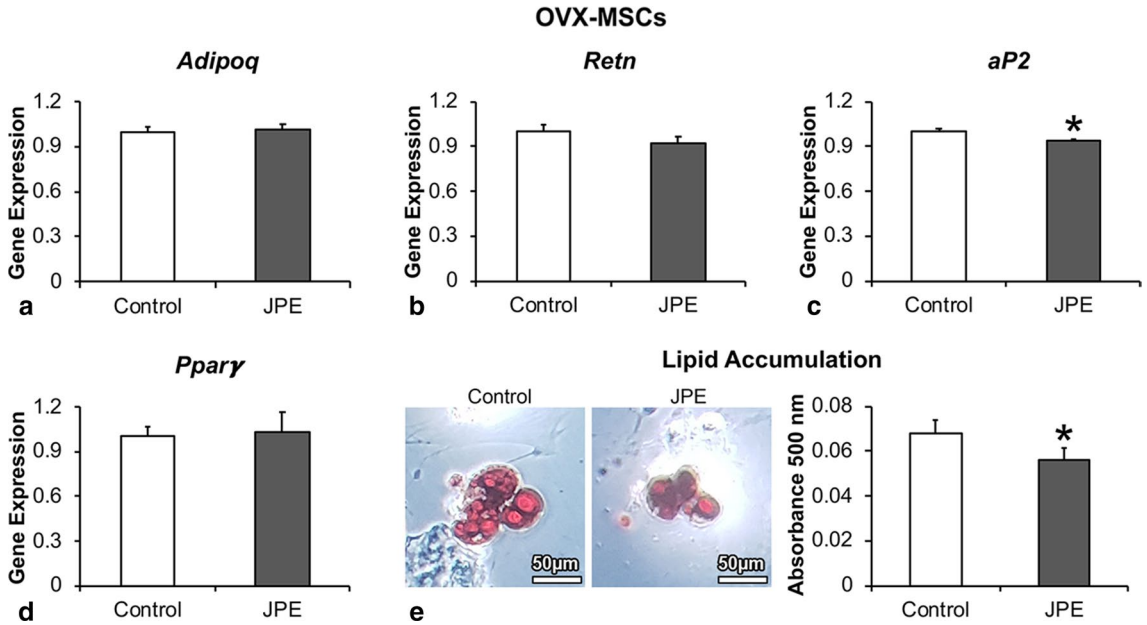

Fig. 5 Effect of JPE on adipocyte differentiation of bone marrow-derived mesenchymal stromal cells from osteoporotic rats (OVX-MSCs). Gene expression of the adipocyte markers adiponectin (*Adipoq*, **a**), resistin (*Retn*, **b**), adipocyte protein 2 (*aP2*, **c**), and peroxisome proliferator-activated receptor gamma (*Pparγ*, **d**) on day 5, and quantification of lipid accumulation (**e**) on day 21 of OVX-MSCs cultured in adipogenic medium without (Control) or with 0.25 µg/ml of JPE. The data of gene expression ($n=3$) and lipid accumulation ($n=5$) are presented as mean ± standard deviation. Asterisks (*) indicate statistically significant difference between Control and JPE (Student's *t* test, $p \leq 0.05$)

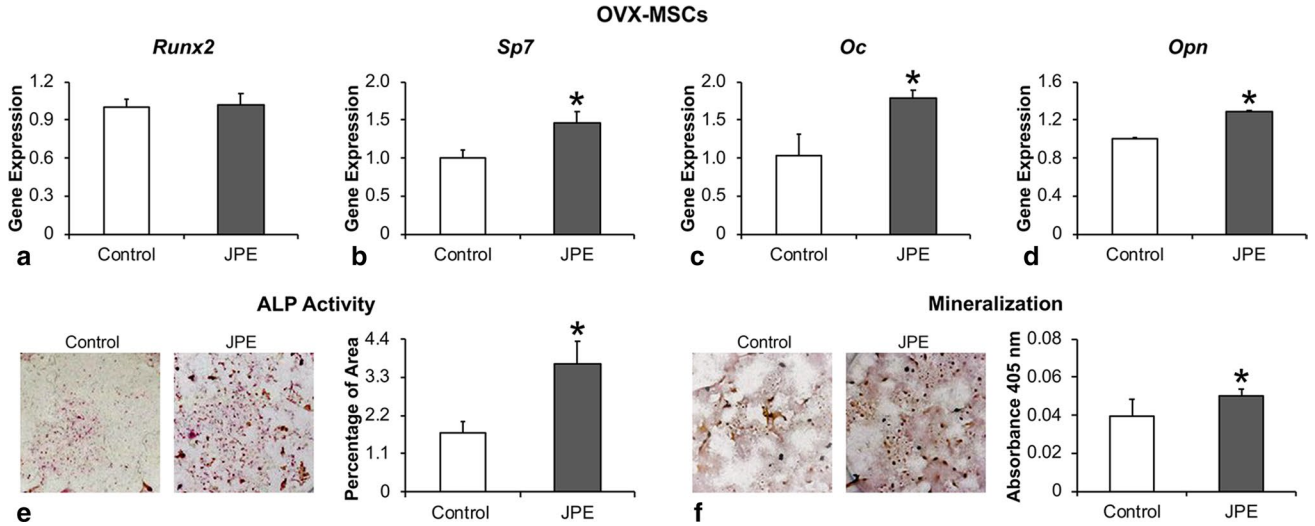

Fig. 6 Effect of JPE on osteoblast differentiation of bone marrow-derived mesenchymal stromal cells from osteoporotic rats (OVX-MSCs). Gene expression of the osteoblast markers runt-related transcription factor 2 (*Runx2*, **a**), osterix (*Sp7*, **b**), osteocalcin (*Oc*, **c**) and osteopontin (*Opn*, **d**) on day 5 and quantification of alkaline phosphatase (ALP) activity on day 10 (**e**) and extracellular matrix mineralization on day 21 (**f**) of OVX-MSCs cultured in osteogenic medium without (Control) or with 10 µg/ml of JPE. The data of gene expression ($n=3$), ALP activity ($n=5$) and extracellular matrix mineralization ($n=5$) are presented as mean ± standard deviation. Asterisks (*) indicate statistically significant difference between Control and JPE (Student's *t* test, $p \leq 0.05$)

this experimental model a reliable source of MSCs from an osteoporotic environment.

Among the several natural compounds, JPE was selected due to its beneficial properties such as anti-inflammatory, antioxidant and mainly anti-obesity features, which include regulation of lipid accumulation and anti-adipogenic effects [15]. Concentrations ranging from 0.25 to 250 µg/ml have been used to investigate the effects of JPE in several cell lines, including HSC-3 (human oral squamous cell carcinoma), L929 (mouse fibroblast), U251 (human glioblastoma) and VERO (green monkey epithelial kidney cell) [10, 42, 43]. Among three different concentrations of JPE used in this study, we selected 0.25 µg/ml to evaluate both adipocyte and osteoblast differentiation of MSCs as it inhibited lipid accumulation and enhanced ALP activity. Under osteoporotic conditions, the most efficient concentration to inhibit lipid accumulation was 0.25 µg/ml, while 10 µg/ml of JPE elicited a higher ALP activity. We used these parameters because the visualization of lipid accumulation by Oil red O staining is one of the tools to assess adipocyte phenotype, while the Fast Red staining is used to detect ALP activity, a marker of osteoblast differentiation [44, 45].

The reduction of bone formation may be associated with increased bone marrow adiposity, indicating that the progression of differentiation of MSCs into either osteoblasts or adipocytes can be coordinated due to this negative correlation between bone mass and adipogenesis [46]. Considering these aspects, the regulation of lipid metabolism and the anti-adipogenic effect of JPE [15] could modulate not only adipocyte, but also osteoblast differentiation of MSCs under both healthy and osteoporotic conditions. Indeed, JPE was able to downregulate the gene expression of most of the adipocyte markers, as well as lipid accumulation in cell cultures from healthy animals. However, despite that the effect of JPE on osteoblasts was not detected by gene expression, which could be due to the fact that mRNA level was evaluated only on day 5, such effect was clearly demonstrated by the increase in the parameters related to the osteoblast phenotype, ALP activity and extracellular matrix mineralization. Under osteoporotic condition, JPE downregulated the gene expression of *aP2*, but did not affect the other evaluated genes; however, it was able to negatively regulate the adipocyte phenotype development, as shown by reduced lipid accumulation. In addition, JPE upregulated the expression of the majority of the evaluated osteoblast marker genes, as well as the parameters related to osteoblast phenotype development. Thus, these results corroborate our hypotheses and showed that JPE is able to inhibit adipocyte differentiation as well as to stimulate osteoblast differentiation, both under healthy and osteoporotic conditions.

The bioactive compounds of the jabuticaba, mainly from its peel, include polyphenols such as anthocyanins, gallic acid, ellagic acid, quercetin and epicatechin, which have shown antioxidant properties and therapeutic functions [47]. Moreover, the effect of JPE on lipid metabolism is also related to the high concentration of polyphenols. Indeed, it has been shown that the high levels of anthocyanins of the extract contributed to the decrease of downregulated lipid accumulation in adipocytes and also the expression of tumor necrosis factor-alpha (NF-κB), a cytokine that regulates the

adipocyte metabolism and inhibits osteoblast differentiation [48–50]. Thus, the anthocyanins present in JPE could be related to decreased adipocyte differentiation and increased osteoblast differentiation of MSCs and OVX-MSCs reported here. Additionally, the NF-κB and sirtuin type 1 deacetylase (Sirt1) have been described as possible targets of the polyphenol-rich foods, where the polyphenols promote cell differentiation and bone formation through inhibition of NF-κB and activation of Sirt1 [51–53].

In conclusion, our results demonstrated that, in general, JPE inhibits adipocyte differentiation of MSCs with more pronounced effects in cells from healthy than osteoporotic rats. Furthermore, JPE increases osteoblast differentiation, exhibiting a slightly higher osteogenic potential on MSCs from osteoporotic compared to healthy condition. These findings can be useful to drive further studies on the therapeutic use of JPE in both prevention and treatment of osteoporosis, since its high availability and low cost could generate clinical approaches accessible to a large portion of the population.

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Author contributions ATPS and GPF carried out the experiments, analyzed the data and drafted the manuscript. HBL and GGCT analyzed the data and revised the manuscript. AGT and MRMJ synthesized and provided JPE, contributed to the design of the study and revised the manuscript. ALR and MMB conceived, designed and supervised the study, analyzed the data and drafted the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All animal experiments were approved by the Committee of Ethics in Animal Research of the School of Dentistry of Ribeirão Preto, University of São Paulo (CEUA # 2018.1.30.58.8) and followed all institutional, national and international guidelines for the care and use of animals.

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