Morinda officinalis polysaccharide enable suppression of osteoclastic differentiation by exosomes derived from rat mesenchymal stem cells

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ABSTRACT

Context: Morinda officinalis F.C. How. (MO) (Rubiaceae) can strengthen bone function. Objective: To examine the functional mechanism and effect of MO polysaccharides (MOPs) in rats with glucocorticoid-induced osteoporosis (GIOP).

Materials and methods: Rats with GIOP were treated with 5, 15 or 45 mL/kg of MOP [n = 15 for each dose, intraperitoneal (i.p.) injection every other day for 8 weeks]. The body weight of rats and histomorphology of bone tissues were examined. Bone marrow mesenchymal stem cells (BMMCs)-derived exosomes (Exo) were collected and identified. Bone marrow-derived macrophages (BMMs) were induced to differentiate into osteoclasts and treated with BMMC-Exo for in vitro studies.

Results: MOP reduced the body weight (5, 15, or 45 mg/kg MOP vs. phosphate-buffered saline: 8%, 15% and 25%, p < 0.01), elevated the bone volume to tissue volume (BV/TV), mean trabecular thickness (Tb.Th), mean trabecular number (Tb.N) and mean connectivity density (Conn.D) (40–86%, p < 0.01), decreased the mean trabecular separation/spacing (Tb.Sp) (22–37%, p < 0.01), increased the cortical bone continuity (35–90%, p < 0.01) and elevated RUNX family transcription factor 2 and RANK levels (5–12%, p < 0.01), but suppressed matrix metalloproteinase 9 and cathepsin K levels (9–20%, p < 0.01) in femur tissues. BMMC-Exo from MOP-treated rats (MOP-Exo) suppressed osteoclastic differentiation and proliferation of BMMs. The downregulation of microRNA-101-3p (miR-101-3p) or the upregulation of prostaglandin-endoperoxide synthase 2 (PTGS2) blocked the functions of MOP-Exo.

Discussion and conclusions: MOP inhibits osteoclastic differentiation and could potentially be used for osteoporosis management. This suppression may be enhanced by the upregulation of miR-101-3p or the inhibition of PTGS2.

Introduction

Osteoporosis (OP) is caused by an imbalance between bone formation by osteoblasts and bone resorption by osteoclasts, leading to low bone mass and microarchitectural disruption of bone tissues that may result in fractures (Eastell and Szulc 2017). Increased differentiation, activation and survival of osteoclasts, mainly induced by receptor activator of nuclear factor-kappa B (RANK) and its ligand RANKL, plays a central role during the pathogenesis of OP (Kanis et al. 2019; Fischer and Haffner-Luntzer 2022). OP can be classified into primary OP, which is mainly caused by menopause, and secondary OP, which is mainly caused by glucocorticoid intake, termed glucocorticoid-induced OP (GIOP) (Soriano et al. 2014; Zhang Z et al. 2016). Continuous glucocorticoid intake causes rapid bone loss and increased fracture risk within 3–6 months of the initiation (Compston 2018). Excessive glucocorticoid usage induces bone demineralization with significant changes in spatial heterogeneities of bone at the microscale (Cheng et al. 2022). Major pharmacological treatments for postmenopausal and senile OP include calcium, active vitamin D supplementation and bisphosphonates and are also used to treat GIOP (Soriano et al. 2014). However, these treatments can only reduce non-vertebral fracture risk but cannot restore lost bone (Appelman-Dijkstra and Papapoulos 2015), and may cause adverse reactions, such as osteonecrosis of the jaw (Pozzi et al. 2007) and gastroesophageal irritation (Abrahamsen 2010). More research is needed to develop tailored therapies for GIOP.

The medicinal plant Morinda officinalis F.C. How. (MO) (Rubiaceae) and its root are traditional herbal medicines used as tonics for the treatment of impotence, OP, depression and inflammatory diseases due to their ability to nourish the kidney, strengthen the bone, and improve immunity (Zhang JH et al. 2018). Its polysaccharides are the major chemical compounds isolated from the herb (Zhang JH et al. 2018). Morinda officinalis polysaccharide (MOP) possesses anti-osteoporotic capabilities. In a study, its treatment in ovariectomized rats prevented the deterioration of the rats’ trabecular microarchitecture and reduced bone turnover markers (Zhang D et al. 2020). However, the effect of MOP on GIOP remains largely unknown. To explore the effect of MOP in disease development, a rat model of GIOP was induced and treated with MOP in this study. Exosomes (Exo) are a class of nanosized extracellular vesicles of endocytic origin that exert crucial functions in intercellular communication (Kouroubanas 2015). Exo can be secreted into the extracellular milieu and absorbed by target cells, where cellular behaviours are regulated by carrying genetic materials, such as microRNAs (miRNAs) and mRNAs, as well as proteins and liquids (Zhang Y et al. 2019). Exo also exert functions...
in the bone’s microenvironment and affect bone metabolism (Li Q et al. 2018; Shane et al. 2019) and regulate the communication between osteoblasts and osteoclasts (Yuan et al. 2018). Exo from bone marrow mesenchymal stem cells (BMSCs) alleviated OP by promoting osteoblast activity (Yang et al. 2019). RANKL can induce osteoclastogenesis, and RANKL-treated bone marrow-derived macrophages (BMMs) have been used to mimic osteoclasts for in vitro studies (Park and Yun 2019). In the present study, BMSC-Exo from GIOP rats were extracted to explore their effect on osteoclastic differentiation of RANKL-treated BMMs to determine whether the MOP treatment affected the biological functions of Exo.

Materials and methods

Establishment of a rat model of GIOP

All animal procedures were approved by the Animal Ethics Committee of Guangzhou Hospital of Integrated Traditional and Western Medicine (approval no. 2020090201) and strictly adhered to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996). A total of 105 male Sprague-Dawley rats (3 months old) were procured from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animals were kept in a specific pathogen-free condition at a constant temperature of 22–25°C in a 12 h light/dark cycle. The rats were provided with drinking water and standard rodent feed including 2.5% casein, 0.8% phosphorus, 1% calcium, 70–80% carbohydrate and 5% fat (Trophic Animal Feed High-tech Co., Ltd., Nantong, China).

After one week of adaptive training, the rats were randomly allocated into control and GIOP groups. The control group rats (n = 15) were treated with 10 mL/kg 0.9% normal saline [intra-peritoneal (i.p.) injection]. GIOP was induced in the rest of the rats (n = 90) by injecting 30 mg/kg dexamethasone-sodium phosphate diluted in 10 mL/kg 0.9% normal saline (i.p.). The injections were performed once every other day from week 1 to week 8. Among the 90 rats with GIOP, 15 were allocated to the model group (GIOP, n = 15). The remaining 75 GIOP rats were further allocated into five groups and injected (i.p.) with 10 mL/kg phosphate-buffered saline (PBS, 0.01 M; n = 15), or injected (i.p.) with 5 mg/kg (n = 15), 15 mg/kg (n = 15) or 45 mg/kg (n = 15) of MOP (Snott Biotechnology Co., Ltd., Shaanxi, China) dissolved in equal doses of PBS, or injected (i.p.) with 15 μg/kg oestradiol (n = 15), a well-known therapy for OP (Levin et al. 2018), as positive controls. All injections were performed once every other day from week 9 to week 16. The weight of the rats on days 1, 4, 11, 18, 25, 32, 39, 48 and 55 was examined. After the final treatment, the rats were euthanized by the administration of 150 mg/kg pentobarbital sodium (i.p.). Their tibias and femurs were collected. A sketch map for the animal grouping and treatment is presented in Figure 1.

Figure 1. A sketch map for animal grouping and treatment.
Micro-computed tomography (CT)

A cone-beam micro-CT system with the associated software (μCT 50, Scanco Medical, Brütisellen, Switzerland) was used to analyse the microstructure of the distal femur. Images were obtained at 70 kV and 200 μA with a spatial resolution of 14.8 mm in all directions, and a 3D image was reconstructed. The volume of interest (VOI) ranged from the trabecular bone region at 2 mm below the peak of the growth plate to the distal femur; 100 CT images were obtained. The bone mass parameters include the following: the bone volume to tissue volume (BV/TV), the mean trabecular thickness (Tb.Th, mm), mean trabecular number (Tb.N, n/mm) and the mean trabecular separation/spacing (Tb.Sp, mm) and the mean connectivity density (Conn.D, 1/mm²).

Immunohistochemistry (IHC)

The femur tissues were fixed and decalcified in 10% ethylene diamine tetraacetic acid (EDTA) for 4 weeks, dehydrated in ethanol (70–100%), cleared with xylene, embedded in paraffin, and cut into 4 μm sections. The sections were dewaxed and reacted with anti-matrix metalloproteinase 9 (MMP-9; 1:200, ab7603, Abcam Inc., Cambridge, MA), anti-RUNX family transcription factor 2 (RUNX2, 1:200, #MA5-41185, Thermo Fisher Scientific, Waltham, MA), anti-RANK (1:200, #MA5-16153, Thermo Fisher Scientific, Waltham, MA) and anti-RANKL (1:200, ab239607, Abcam, Cambridge, MA) or goat anti-mouse IgG (H&L) (HRP) (1:1000, ab6721, Abcam, Cambridge, MA) or goat anti-rabbit immunoglobulin G (IgG) (H&L) (HRP) (1:1000 ab6789, Abcam, Cambridge, MA) at 37 °C for 1 h. The endogenous expression of MMP9, RUNX2, RANK and RANKL in tissues was analysed using the Image-Pro Plus software (Rockville, MD).

Safranin O-fast green staining and haematoxylin and eosin (HE) staining

The femur tissue samples were fixed in 4% paraformaldehyde (PFA) for 24 h. The bones were placed in 10% EDTA for one month of decalcification, embedded with paraffin, prepared as 5 μm sections, and stained using a safranin O-fast green staining kit (G1120, Solarbio, Beijing, China) or an HE staining kit (G1120, Solarbio, Beijing, China) according to the manufacturer’s instructions. The images were captured under the CX43 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) at ×200 magnification.

Extraction of rat BMSCs

BMSCs were extracted from rat femur tissues. The BMSCs were cultured in Dulbecco’s modified Eagle medium (DMEM) at 37 °C with 5% CO₂. The medium was first renewed at 24 h and 48 h, and then renewed once every three days. BMSCs from T3 to T5 segments were collected for subsequent experiments. Expression of CD29, CD34, CD44, CD45 and CD90 in BMSCs was examined by flow cytometry to examine the chondrogenic, adipoigenic and osteogenic differentiation abilities of cells, respectively. The chondrogenic differentiation induction medium contains high-glucose DMEM, 40 μg/mL proline, insulin-transferrin-selenium + premix, 5.33 μg/mL linoleic acid, 50 μg/mL ascorbic acid, 100 nM dexamethasone, 1.25 mg/mL bovine serum albumin (BSA), 10 ng/mL TGFβ3, 1 mM sodium pyruvate and 6.25 μg/mL selenite. The adipoigenic differentiation induction medium contains 10 nM dexamethasone, 0.1 mmol/L 3-isobutyl-1-methylxanthine, 50 μg/mL indomethacin, 10 μg/mL insulin, high-glucose DMEM and 10% foetal bovine serum (FBS). The osteogenic differentiation induction medium contains 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, 10 nM dexamethasone, 10% FBS and high-glucose DMEM. The media were renewed every three days. Adipose cells were stained by oil red O, chondrocytes were stained by Alcian blue, and osteoblasts were stained by Alizarin Red S on the 14th day.

Extraction and identification of BMSC-Exo

To prepare conditioned medium, the BMSCs were cultured in 10% Exo-free FBS-contained DMEM for 48 h. To exclude the dead cells or cell debris, the medium was centrifuged at 300 × g for 10 min, at 2000 × g for 20 min, at 10,000 × g for 30 min, and the supernatant was further ultra-centrifuged at 100,000 × g for 1 h. Thereafter, the particles were washed and re-suspended in PBS, and centrifuged at 100,000 × g for 1 h. The shape of the collected particles was observed using a transmission electron microscope (TEM; #H-7000FA, HITACHI, High-Technologies Corporation, Tokyo, Japan). Nanoparticle tracking analysis (NTA) was performed using the Zetasizer Nano system (Malvern, Worcestershire, UK). The protein expression of CD9 (1:1000, #98327, Cell Signaling Technology (CST), Beverly, MA), CD63 (1:1000, ab217345, Abcam, Cambridge, MA), Flotillin-1 (1:1000, #18634, CST, Beverly, MA) and Calnexin (1:1000, ab133615, Abcam, Cambridge, MA) was examined by western blot analysis.

Cell culture and treatment

BMMs were collected from the femur tissue of rats and cultured in 96-well plates at 1 × 10⁴ cells per well for 24 h. After adherence, the cells were cultured in the medium with or without recombinant rat RANKL protein (100 ng/mL; ab209177, Abcam, Cambridge, MA), Exo, miRNA inhibitor or prostaglandin-endoperoxide synthase 2 (PTGS2) overexpression vector (oe-PTGS2). The medium was refreshed every two days. After six days, the osteoclastic differentiation of BMMs was examined using tartrate-resistant acid phosphatase (TRAP) staining.

Uptake of BMSC-Exo by BMMs

The BMSC-Exo (10 μg) were labelled using a PHK67 green fluorescence kit (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). In short, Exo in PBS were added to 0.5 mL dilution C, and 4 μL PKH67 was added to 0.5 mL dilution C as well. The two mixtures were incubated at 22–25 °C in the dark for 4 min, and then 2 mL 0.5% BSA was used to avoid over-labelling. The labelled Exo were resuspended in 35 mL PBS and centrifuged at 120,000 × g for 70 min to discard the stain residue. Recipient cells (BMMs) were mixed with PKH67-labelled Exo and incubated in the dark for 24 h. The supernatant was discarded, and the cells were washed with PBS and fixed in 10% PFA for 10 min. The nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) in the dark for 2 min. The fluorescence images were captured under a fluorescence microscope (Leica Microsystems, Mannheim, Germany).
**TRAP staining**

TRAP staining was performed according to the instructions of a TRAP kit (BH0020, Powerful Biology, Wuhan, China). In brief, the BMMs were fixed in 4% PFA, cleared in 1% Triton X-100-contained PBS for 30 min, and pre-incubated with donkey serum to block non-specific binding. The cells were incubated with the primary antibodies against cathepsin K (CTSK; 1:100, Abcam, Cambridge, MA) and MMP9 (1:250, ab76003, Abcam, Cambridge, MA) at 4°C overnight, and then with Cy3-labelled donkey anti-rabbit IgG (406402, Biolegend, San Diego, CA) at 37°C in the dark for 1 h. The nuclei were then labelled with DAPI at room temperature for 90 s, and the images were captured under a fluorescence microscope (Leica, Mannheim, Germany).

**Immunofluorescence staining**

The RANKL-induced BMMs were seeded on glass slides, cultured at 37°C for 24 h, fixed in 4% PFA, cleared in 1% Triton X-100-contained PBS for 30 min, and pre-incubated with donkey serum to block non-specific binding. The cells were incubated with the primary antibodies against cathepsin K (CTSK; 1:100, Abcam, Cambridge, MA) and MMP9 (1:250, ab76003, Abcam, Cambridge, MA) at 4°C overnight, and then with Cy3-labelled donkey anti-rabbit IgG (406402, Biolegend, San Diego, CA) at 37°C in the dark for 1 h. The nuclei were then labelled with DAPI at room temperature for 90 s, and the images were captured under a fluorescence microscope (Leica, Mannheim, Germany).

**Cell counting kit-8 (CCK-8) method**

The RANKL-induced BMMs were incubated in 96-well plates (8 x 10³ cells per well) overnight. The medium was then refreshed with doses of Exo added. After 96 h, 10 μL CCK-8 solution (Beyotime Biotechnology Co., Ltd., Shanghai, China) was added to each well for 2 h of incubation at 37°C in the dark. The optical density at 450 nm was read using a multimodal cell imaging reader (Cytation five; BioTek, Winooski, VT).

**Colony formation assay**

The BMMs were trypsinized, washed in PBS, stained with Trypan-blue to calculate the cell counts, and resuspended into 0.5 x 10⁵ cells/mL. Next, 2 mL cell suspension was added to six-well plates and incubated at 37°C. A half of the medium was replenished on day 5, and the medium was discarded on day 14. Thereafter, the cells were washed in PBS, fixed in 4% PFA for 10 min, and stained with crystal violet for 5 min. The cell colonies formed by BMMs were counted under a microscope (DM1000; Leica, Mannheim, Germany) with five random fields of views included.

**Dual luciferase reporter gene assay**

The wild-type (WT) and mutant-type (MT) binding sequences between microRNA-101-3p (miR-101-3p) and PTGS2 mRNA were obtained or designed. The pGL3-basic luciferase reporter plasmid (Promega, Madison, WI) containing the PTGS2 3′-untranslated region fragment and the Renilla plasmid (pRL-TK; Promega, Madison, WI) were co-transfected with mimic negative control (NC) or miR-101-3p mimic (GenePharma Co., Ltd., Shanghai, China) into 293T cells using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA). After 48 h, the luciferase activity in cells was determined using a dual luciferase reporter vector kit (Promega, Madison, WI) according to the manufacturer’s instructions.

**Biotin-RNA pull down assay**

Biotin-labelled miR-101-3p (Bio-miR-101-3p) and the control probe Bio-NC procured from Sangon Biotech Co., Ltd. (Shanghai, China) were transfected into 293T cells. The cells were lysed, and the cell lysates were hybridized with streptavidin-conjugated magnet beads at 4°C overnight. The abundance of PTGS2 mRNA was examined using reverse transcription quantitative polymerase chain reaction (RT-qPCR).

**Microarray analysis**

Total RNA from BMs was extracted and quantified using a NanoDrop™ One/OneC Productivity kit (Thermo Fisher Scientific, Waltham, MA). The RNA integrity was examined using a Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA). After that, 100 ng total RNA and a Quick-Amp labelling Kit (Agilent, Palo Alto, CA) was used to prepare complementary RNA (cRNA). The cRNA was hybridized using the microarray chip hybridization kit (Agilent, Palo Alto, CA) by following the instructions of the GeneChip™ miRNA 3.0 array (Cat No. 902019, Applied Biosystems Inc., Carlsbad, CA) or the GeneChip™ rat gene 2.0ST array (Cat No. 902124, Applied Biosystems, Carlsbad, CA) at 45°C for 16 h. The microarray was placed on a fluidic station FS-450 (Agilent, Palo Alto, CA) for washing and staining. The microarrays were scanned using a GeneChip scanner (Agilent, Palo Alto, CA). The CEL file was imported into the Partek Genomics Suite™ 6.6 Program (Partek, St. Louis, MO), and the probe sets were normalized by the Robust Multiarray Average method.

**RT-qPCR**

Total RNA from tissues or cells was extracted using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA). The RNA was reverse-transcribed into cDNA using a cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA) at 37°C for 1 h and 85°C for 5 min. Gene amplification was performed using PowerTrack™ SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA) on a StepOnePlus thermal cycler (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions. Relative PTGS2 or miR-101-3p expression was examined using the 2-DDCq method (Livak and Schmittgen 2001). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as the endogenous controls. The primers are listed in Table 1.

**Western blot analysis**

BMMs were trypsinized and lysed in radio-immunoprecipitation assay (RIPA) cell lysis buffer on ice to collect total protein. The

| Table 1. Primers for RT-qPCR. |
|-----------------------------|
| Symbol | Forward (5′–3′) | Reverse (5′–3′) |
| CTSK | CCGTGTTGACCTTGTGCTCTA | CTATGGGCAACAAAGAGGG |
| MMP9 | GCCGGAGATCATGTCGAAA | GTGTTGAAACTCAGACC |
| PTGS2 | CCTACGTGATGCAGCAAAATCC | GGGTGGGCTTCAGAGATA |
| GAPDH | TGCATCTCTTGGAAGTCGAGC | ACCAGCCTTCCCTACAGC |
| miR-101-3p | TACGTTTACAGTGTCTGAT | GAATGTCGTCATGCTC |
| U6 | CCCTCGTTGCCGACACAT | TTTGCGTGTCTCGACG |
proteins and 628.96 vs. 641.56, column factor $p < 0.01$, $F$ [2, 12] = 110.7, all $p < 0.05$). The oestradiol (15 g/kg) treatment slightly elevated the body weight of rats (PBS vs. oestradiol, $p < 0.01$, $F$ [2, 6] = 34.92) and increased by GIOP-Exo (59.37%, $p < 0.01$, $F$ [2, 6] = 76.78, $F$ [2, 6] = 34.92) (Figure 3(F,G)).

**MOP treatment alleviates OP symptoms in rats with GIOP**

The experiments above showed that GIOE-Exo can promote the growth and differentiation of osteoclasts that may exacerbate OP. MOP has shown potential by promoting osteoblast proliferation, thereby alleviating OP symptoms (Yan et al. 2019; Zhang D et al. 2020), but its effect on osteoclasts has yet to be explored. In this study, the rats with OP were treated with different doses (5, 15 and 45 mL/kg) of MOP, and those treated with oestradiol were positive controls (Figure 4(A)). We found that treatment by MOP at 15 or 45 mg/kg significantly reduced the body weight of GIOP rats (PBS vs. 15 mg/kg MOP: 628.96 vs. 540.91; PBS vs. 45 mg/kg MOP: 628.96 vs. 484.3, column factor $F$ [4, 630] = 110.7, all $p < 0.01$). The 5 mg/kg treatment did not significantly affect the body weight of rats (PBS vs. 5 mg/kg MOP: 628.96 vs. 641.56, $p > 0.05$). The oestradiol (15 mg/kg) treatment slightly elevated the body weight of rats (PBS vs. oestradiol, 628.96 vs. 641.56, $p > 0.05$), though there was no significant difference (Figure 4(B)). The OP symptoms in rats, based on the bone mass parameters determined by micro-CT analysis, were significantly alleviated by MOP or oestradiol treatment, as manifested by increased BV/TV (38–105%, $p < 0.01$, $F$ [4,
Figure 2. Establishment of the rat model of GIOP and extraction of BMSC-Exo. (A) Weight of rats on day 1, 4, 11, 18, 25, 32, 39, 48 and 55 after glucocorticoid or normal saline treatment; (B) bone mass parameters of rats determined by micro-CT analysis; (C, D) morphological changes in rat tibial tissues examined by HE staining and safranin O-fast green staining; (E) morphology of BMSCs extracted from rat femur tissues observed under a light microscope; (F) expression of CD90, CD29, CD34, CD44 and CD45 in the isolated cells determined by flow cytometry; (G) osteogenic, adipogenic and chondrogenic differentiation abilities of the isolated cells determined by Alizarin Red S staining, oil Red O staining and Alcian Blue staining, respectively; (H) a sketch map for the extraction of Exo from the conditioned medium of BMSCs using gradient centrifugation; (I) size distribution of the collected particles determined by NTA; (J) shape of the collected particles under the TEM; (K) expression of the Exo-specific marker proteins CD63, CD9 and Flotillin-1 and the cytoplasm marker protein Calnexin in the collected particles examined by western blot analysis. At least three independent experiments were performed. In animal studies, n = 15 in each group. Differences were compared by two-way ANOVA (A) or the unpaired t-test (B and D). **p < 0.01.
70% = 83.48), Tb.Th (32–99%, p < 0.01, F [4, 70] = 82.45), Tb.N (38–94%, p < 0.01, F [4, 70] = 70.41) and Conn.D (31–100%, p < 0.01, F [4, 70] = 71.96) and reduced Tb.Sp (21–39%, p < 0.01, F [4, 70] = 53.25) (Figure 4(C)). In addition, the HE and safranin-O-fast green staining results showed that the cortical bone continuity in rats was increased, whereas the number of osteoblasts was significantly increased after MOP treatment (35–90%, p < 0.01, F [4, 70] = 85.35 (Figure 4(D,E)). The IHC results show...
that osteogenesis-related RUNX2 (38–118%, \(p < 0.01, F_{[4, 70]} = 101.1\)) and RANK levels (41–99%, \(p < 0.01, F_{[4, 70]} = 68.51\)) in rat femur tissues were increased, whereas the levels of MMP9 (24–44%, \(p < 0.01, F_{[4, 70]} = 65.80\)) and CTSK (18–44%, \(p < 0.01, F_{[4, 70]} = 65.90\)) were reduced following MOP or oestradiol treatment (Figure 4(F–I)). These results show that MOP or oestradiol treatment can promote osteoblastic differentiation and suppress osteoclastic differentiation.

**BMSC-Exo extracted from MOP-treated GIOP rats inhibit osteoclast differentiation of RANKL-induced BMMs**

To explore the effect of MOP treatment on the function of BMSC-Exo from rats in osteoclast differentiation, the BMSCs from GIOP rats were extracted following different doses of MOP treatment. After gradient centrifugation, the Exo of the BMSCs were then extracted and termed L-MOP-Exo (Exo collected from rats treated with 5 mL/kg of MOP), M-MOP-Exo (Exo collected from rats treated with 15 mL/kg of MOP) and H-MOP-Exo (Exo collected from rats treated with 45 mL/kg of MOP). BMMs were first treated with 50 \(\mu\)g/mL Exo and then treated with RANKL to induce osteoclast differentiation. Following RANKL induction, we found that the MOP-Exo significantly reduced the amount of BMMs that differentiated into osteoclasts (24–40%, \(p < 0.01, F_{[3, 8]} = 14.34\)) (Figure 5(A)). As shown in the immunofluorescence staining, the levels of CTSK (28–60%, \(p < 0.01, F_{[3, 16]} = 47.78, F_{[3, 16]} = 29.91\)) and MMP9 (18–43%, \(p < 0.01, F_{[3, 16]} = 47.78, F_{[3, 16]} = 29.91\)) in cells significantly decreased after MOP-Exo treatment (20–60%, \(p < 0.01\)) (Figure 5(B,C)). Furthermore, the MOP-Exo treatment reduced the proliferation (24–39%, \(p < 0.01, F_{[3, 8]} = 8.722\)) and colony-forming ability (27–51%, \(p < 0.01, F_{[3, 8]} = 21.66\)) of the BMMs (Figure 5(D,E)).
MOP treatment reduces PTGS2 expression in GIOP rats and BMSC-Exo

To explore the molecular mechanism, a microarray analysis was conducted to analyse differentially expressed genes (DEGs) in BMMs after MOP-Exo treatment. Consequently, 102 DEGs were identified, and the top 30 DEGs were shown in the heatmap (Figure 6(A,B)). The candidate genes regulated by MOP were predicted using the bioinformatic system (http://herb.ac.cn/) and compared with the DEGs; PTGS2 was identified (Figure 6(C)). We then examined the expression of PTGS2 in rat bone tissues. The RT-qPCR and western blot assay results indicate that both the mRNA and protein levels of PTGS2 were elevated (mRNA: 225%, p < 0.01, F [5, 12] = 34.87; protein: 128%, p < 0.01, F [5, 12] = 23.89) in the bones of rats with GIOP, whereas the MOP treatment significantly reduced PTGS2 expression (mRNA: 21–31%, p < 0.01, F [5, 12] = 34.87; protein: 26–45%, p < 0.01, F [5, 12] = 23.89) in rat models (Figure 6(D,E)). Furthermore, RANKL treatment increased the expression of PTGS2 in BMMs (mRNA: 397%, p < 0.01, F [8, 18] = 53.97; protein: 155%, p < 0.01, F [8, 18] = 33.17). Moreover, treatment of GIOP-Exo further increased PTGS2 expression in BMMs (mRNA: 397%, p < 0.01, F [8, 18] = 53.97; protein: 155%, p < 0.01, F [8, 18] = 33.17) but upon treatment of MOP-Exo, the PTGS2 levels in cells were decreased (mRNA: 24–59%, p < 0.01, F [8, 18] = 53.97; protein: 19–48%, p < 0.01, F [8, 18] = 33.17) (Figure 6(F,G)). Based on these results, we hypothesize that MOP reduces PTGS2 in rats and in Exo, thereby suppressing osteoclastic differentiation.

Overexpression of PTGS2 blocks the inhibiting effect of H-MOP-Exo on osteoclastic differentiation

To test our hypothesis, oe-PTGS2 was transfected into BMMs. The western blot analysis showed that PTGS2 was upregulated
The BMMs were then treated with H-MOP-Exo and RANKL. Following the overexpression of PTGS2, the number of BMMs that differentiated into osteoclasts was increased, as indicated by the number of multinucleated cells in the TRAP staining (NC vs. oe-PTGS2: 26.59 vs. 38.44, \( p < 0.01, F[2, 2]=2.115 \)) (Figure 7(B)). Furthermore, the immunofluorescence staining results showed that the expression of CTSK and MMP9 in cells was restored by oe-PTGS2 (147–415%, \( p < 0.01, \)) column factor \( F[1, 8]=305.8, F(1, 8)=42.16 \) (Figure 7(C,D)). The proliferation activity and colony-forming ability of the BMMs were re-examined. Likewise, the proliferation (32%, \( p < 0.01, F[2, 2]=2.551 \)) and colony formation (81%, \( p < 0.01, F[2, 2]=3.214 \)) of BMMs was significantly increased by oe-PTGS2 overexpression (Figure 7(E,F)). These rescue experiments show that mediation by MOP-Exo inhibits PTGS2.

miR-101-3p expression is significantly elevated in BMSC-Exo following MOP treatment

Since Exo often carry miRNAs for intercellular communication, we surmised that the MOP-Exo affect the expression of PTGS2 by communicating with specific miRNA(s). Therefore, a miRNA microarray analysis was also conducted to screen differentially expressed miRNAs in BMMs after MOP-Exo treatment. A total of 146 differentially expressed miRNAs were identified. The top 30 differentially expressed miRNAs were shown in the heatmap (Figure 8(A,B)). miRNAs that can regulate PTGS2 mRNA were predicted using bioinformatic systems TargetScan (http://www.targetscan.org/vert_72/) and miRwalk (http://mirwalk.umm.uni-heidelberg.de/). We plotted another Venn diagram, and miR-101-3p was identified and shown to be intersected (Figure 8(C)).

The expression of miR-101-3p in rat bone tissues was examined and was found to be decreased (84%, \( p < 0.01, F[5, 84]=1153 \)) in rats with GIOP compared to the controls but increased in GIOP rats following MOP treatment (106–384%, \( p < 0.01, F[5, 84]=1153 \)) (Figure 8(D)). In addition, the expression of miR-101-3p was significantly decreased (76%, \( p < 0.01, F[8, 18]=182.5 \)) in BMMs after RANKL treatment. Treatment of GIOP-Exo led to a greater decrease (54%, \( p < 0.01, F[8, 18]=182.5 \)) whereas treatment of MOP-Exo led to an increase (172–536%, \( p < 0.01, F[8, 18]=182.5 \)) in the expression of miR-101-3p in the RANKL-treated BMMs (Figure 8(E)). Furthermore, data in the RNA-locate system (http://www.rna-society.org/rnalocate/index.html) suggest a broad distribution of miR-101-3p in Exo (Figure 8(F)). Collectively, these results suggest that MOP treatment increases the expression of exosomal miR-101-3p to suppress PTGS2 expression and reduce osteoclast differentiation.

The luciferase reporter assay was performed to validate the binding between miR-101-3p and PTGS2 mRNA. Transfection of the miR-101-3p mimic led to a significant decline in the luciferase activity of PTGS2-WT (73%, \( p < 0.01, \)) column factor \( F[1, 8]=171.2 \) but not PTGS2-MT in 293T cells (Figure 7(G,H)). In addition, Bio-miR-101-3p was introduced into BMMs for the

Figure 6. MOP treatment reduces PTGS2 expression in GIOP rats and BMSC-Exo. (A, B) DEGs in BMMs after MOP-Exo treatment identified by microarray analysis; (C) a Venn diagram for the intersection between DEGs identified above and the potential genes regulated by MOP; mRNA (D) and protein (E) expression of PTGS2 in BMSC-Exo. (F,G) mRNA (F) and protein (G) expression of PTGS2 in rat bone tissues examined by RT-qPCR and western blot analysis. At least three independent experiments were performed. Differences were compared by one-way ANOVA (D–G). * \( p < 0.01. \)
Discussion

It is estimated that 1–2% of the population receive long-term glucocorticoid treatment (Fardet et al. 2011; Overman et al. 2013; Silverman et al. 2015), and approximately 50% of patients receiving long-term glucocorticoid treatment develop OP (Ioannidis et al. 2014). Studies have reported MOP’s potential in strengthening bone function. In this study, we confirmed the alleviating effect of MOP treatment on rats with GIOP. We found that MOP treatment grants rat BMSC-Exo the ability to inhibit osteoclast differentiation.

As Exo can be safely applied and possesses potent osteogenic abilities, using Exo to reverse OP is promising (Xie X et al. 2020). Different studies have shown a relationship between the application of Exo and osteogenic differentiation of MSCs or a relationship with the activity of osteoblasts. For instance, Exo from osteogenic MSCs triggered osteogenic differentiation of undifferentiated MSCs both in vitro and in vivo (Narayan K et al. 2018). Exo from endogenous BMSCs also induce osteogenic differentiation (Chen et al. 2017). Exo can influence the maturation and activity of osteoclasts as well. Exo derived from endothelial progenitor cells promoted the migration and osteoclastic differentiation of BMSCs (Cui et al. 2016). Exo collected from pathological conditions, such as those derived from tumour cells (Inder et al. 2014; Guo et al. 2019), strongly promoted osteoclastogenesis or proliferation and differentiation of osteoclasts. In this study, treatment of BMSC-Exo from control rats reduced osteoclastic differentiation of RANKL-induced BMSCs, whereas GIOP-Exo significantly elevated osteoclastic differentiation and growth of BMSCs. Similarly, a study reported that serum-derived Exo from aging patients with osteopenia facilitated osteoclastic activity and bone remodelling, but the Exo from normal volunteers protected bone health by facilitating bone cell adhesion and reducing ageing-associated oxidative stress (Xie Y et al. 2018).

As mentioned previously, MOP improves kidney function and reduces OP symptoms. Furthermore, according to traditional Chinese medicine theory, a deficiency in kidney essence is related to the development of OP (Zhang JH et al. 2018). In an animal study, treatment using an aqueous extract of MO’s root increased hind limb thickness, tibial failure load and bone mineral density (BMD) of sciatic-neurectomized mice, which suppressed bone resorption and promoted bone formation in vivo (See et al. 2005). In the present study, we observed that MOP treatment in rats treated with dexamethasone improved bone mass parameters, alleviated OP symptoms, reduced the number of osteoclasts, increased the levels of osteogenic RUNX2 and RANK, and reduced the levels of MMP9 and CTSK, factors related to osteolysis. These results are consistent with the findings from a previous study, which reports that MO treatment increased BMD and improved microarchitecture by regulating TRAP, alkaline phosphatase and c-terminal telopeptides of type I collagen (CTX-I) in rats with GIOP (Xia et al. 2019). Since mRNA and protein expression of PTGS2 in cells and found that they were elevated significantly upon the inhibition of exosomal miR-101-3p (mRNA: 235%, \(p < 0.01, F [2, 2] = 46.24\); protein: 80%, \(p < 0.01, F [2, 2] = 2.56\) (Figure 9(B,C)), and RANKL-treated BMSCs that differentiated into osteoclasts were increased (H-MOP-Exo/NC vs. H-MOP-Exo/inhibitor: 23.42 vs. 38.71, \(F [2, 2] = 2.189\), Figure 9(D)). Likewise, the downregulation of miR-101-3p in Exo restored the colony-forming ability of the BMMs (42%, \(p < 0.01, F [2, 2] = 2.601\) (Figure 9(E)).

Downregulation of miR-101-3p in MOP-Exo increases osteoclast differentiation and growth of BMSCs

The results above suggest that MOP treatment elevates the expression of exosomal miR-101-3p and suppresses PTGS2 expression, thereby inhibiting osteoclast differentiation. To validate the role of miR-101-3p in osteoclast differentiation, miR-101-3p inhibitors were transfected into the BMSCs from H-MOP-treated rats. The knockdown efficacy was 79% \((p < 0.01, \text{column factor } F [1, 8] = 2475)\). The BMSC-Exo were collected, termed H-MOP-Exo/inhibitor. The H-MOP-Exo/inhibitor greatly suppressed the expression of, in which reduced miR-101-3p was detected as well \((87\%, p < 0.01, \text{column factor } F [1, 8] = 2475)\) (Figure 9(A)). The BMMs were treated with either the H-MOP-Exo/inhibitor or the control H-MOP-Exo/NC. We observed the
Figure 8. miR-101-3p expression is significantly elevated in BMSC-Exo following MOP treatment. (A, B) Differentially expressed miRNAs in BMMs after MOP-Exo treatment identified by microarray analysis; (C) a Venn diagram for the intersected miRNA between the differentially expressed miRNAs identified above and the upstream regulatory miRNAs of PTGS2 mRNA from two bioinformatic systems; (D) expression of miR-101-3p in rat bone tissues examined by RT-qPCR; (E) expression of miR-101-3p in BMMs determined by RT-qPCR; (F) distribution of miR-101-3p in the microvesicles from different sources of human tissues; (G) putative (WT) binding sequence between miR-101-3p and PTGS2 mRNA and the MT sequence designed for luciferase assay; binding relationship between miR-101-3p and PTGS2 validated via luciferase (H) and Biotin-labelled RNA pull down (I) assays. At least three independent experiments were performed. Differences were compared by one-way ANOVA (D, E and I) or two-way ANOVA (H). **p < 0.01.

Figure 9. Downregulation of miR-101-3p in MOP-Exo increases osteoclast differentiation and growth of BMMs. (A) The BMSCs from GIOP rats after H-MOP treatment were collected and transfected with miR-101-3p inhibitor; expression of miR-101-3p in the BMSCs and the Exo examined by RT-qPCR; (B, C) mRNA (B) and protein (C) expression of PTGS2 in BMMs examined by RT-qPCR and western blot analysis; (D) the portion of multinucleated cells in the Exo-pre-treated and RNAKL-induced BMMs in the setting of miR-101-3p inhibition determined by TRAP staining; (E) colony formation ability of the BMMs examined by the colony formation assay. At least three independent experiments were performed. Differences were compared by the unpaired t-test (C–E) or two-way ANOVA (A). **p < 0.01.
GIOP-Exo promotes osteoblast differentiation of RANKL-treated BMMs in vitro, we focussed on the effect of MOP treatment on the function of GIOP-Exo. A natural oligosaccharide from MO decreased RANKL-induced osteoclast formation and bone resorption (Hong et al. 2017). We observed that the MOP-Exo significantly reduced the levels of MMP9 and CTSK and reduced osteoclastic differentiation and growth of the RANKL-induced BMMs.

We then explored the molecular mechanism. Exosomal miRNAs are increasingly thought to be major regulators of gene expression and control the differentiation and activities of osteoclasts (Xie X et al. 2020; Hensley and McAlinden 2021). With the aid of bioinformatics prediction and microarray analyses, the PTGS2 gene was identified and found to be downregulated, and miR-101-3p was upregulated in both MOP-treated rats and MOP-Exo. Since RANKL treatment reduces miR-101-3p in BMMs, especially when the BMMs were pre-treated with GIOP-Exo, we surmised that the MOP treatment will increase the amount of miR-101-3p in rats and in the Exo and lead to the downregulation of PTGS2. The upregulation of miR-101-3p promoted osteogenic differentiation of BMSCs by downregulating Dickkopf 1 (Xiang et al. 2020), but more importantly, the downregulation of miR-101-3p was found to be associated with promoting osteoclast maturation, and miR-101-3p reduced osteoclast differentiation in ovariectomized mice (Li J et al. 2019). We confirmed a direct binding relationship between miR-101-3p and PTGS2 mRNA. COX2 (PTGS2) selective inhibitors were used as medications to relieve musculoskeletal pain (O’Connor and Lysz 2008). In postmenopausal women, the inhibition of COX2 (PTGS2) also increased BMD (Salari and Abdollahi 2009). PTGS2 promotes the maturation of osteoclasts through a COX2-prostaglandin E2 pathway (Lu et al. 2017). Sinomenii Caulis extract, another traditional Chinese medicine, reduced the levels of factors related to osteoclasts, including PTGS2 and MMP9, and alleviated OP symptoms (Li et al. 2020). Our rescue experiments validated that either the downregulation of miR-101-3p or the upregulation of PTGS2 blocked the suppressive effect of MOP-Exo on osteoclastic differentiation of BMMs, which is of clinical significance.

Conclusions
This study demonstrates that MOP treatment can alleviate OP symptoms and improve bone recovery in rats with GIOP. MOP grants BMSC-Exo of rats the ability to suppress osteoclastic differentiation by upregulating miR-101-3p and downregulating PTGS2 (Figure 10); thus, MOP may be applicable to the treatment of GIOP.

Disclosure statement
The authors declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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