Iraqi Journal of Science, 2022, Vol. 63, No. 12, pp: 5166-5185
DOI: 10.24996/ijs.2022.63.12.8

ISSN: 0067-2904

Proinflammatory Activation of Osteoclasts Due to High Prolactin Level

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Received: 19/1/2022 Accepted: 13/6/2022 Published: 30/12/2022

Abstract

High concentrations of prolactin (PRL) during the lactation period have an essential role in milk production by mammary glands stimulation. PRL may have an impact on calcium regulation and bone mineral density. We investigated if the PRL concentration during the lactation period could influence osteoclast (OC) activation and bone mineral density (BMD). In vivo, the Calcium Detection Assay, and ELISA were used to detect serum calcium, PRL, and inflammatory cytokines, respectively. BMD was evaluated by µ-CT in six months old female mice during lactation. The osteoclast (OC) activity was detected by Tartrate-resistant acid phosphatase (TRAP), Immunohistochemistry (IHC), and hematoxylin and eosin (H&E). In vitro, osteoclast differentiation, resorption and their activity markers TRAP, Matrix metallopeptidase 9 (MMP-9), Cathepsin K (CTSK), C-reactive protein (CRP), Receptor activator of nuclear factor kappa-B (RANK) and inflammatory cytokines were measured in osteoclasts stimulated with recombinant prolactin protein (rPRL) or with an anti-prolactin blocker. We found that serum calcium, PRL, and inflammatory markers were increased. BMD was significantly reduced in lactating mice; TRAP activity was increased and tubercular was reduced in lactating mice compared to normal mice. In vitro, the osteoclast number, resorption, and activation markers TRAP, MMP-9, CTSK, CRP, and RANK were significantly increased after treatment with rPRL protein, but not in osteoclasts treated with anti-prolactin receptor antibody and rPRL. The gene expression of TNF-α, IL-6, and Monocyte chemoattractant protein-1 (MCP-1) but not IL-1b were significantly increased in osteoclasts with PRL treatment compared to the untreated osteoclasts. Taken together, the high level of PRL could activate osteoclasts and proinflammatory cytokines expression which reduce BMD in the lactation period.

Keywords: prolactin, bone mineral density, osteoclasts

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التنشيط الالتهابي لخلايا هادمات العظم بسبب ارتفاع مستوى البرولاكتين

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الخلاصة

استهدفت الدراسة الحالية توضيح فيما إذا كان PRL اثناء الرضاعة يمكن أن يؤثر على تنشيط ناقضة العظم Osteoclast وBMD. تم استخدام اختبار الكشف عن الكالسيوم والاليزا للكشف عن الكالسيوم في الدم و PRL والسيتوكينات الالتهابية. تم تقييم BMD بواسطة جهاز μ−CT في إناث الفئران البالغة من العمر ستة أشهر أثناء الرضاعة. تم الكشف عن نشاط Osteoclast بواسطة TRAPو IHC و H&E في المختبر. تم قياس تمايز Osteoclast والارتشاف وعلامات نشاطها TRAP و MMP−9 و CTSK و CRP و RANK والسيتوكينات الالتهابية في Osteoclast التي تم تحفيزها باستخدام بروتين البرولاكتين المؤتلف (rPRL) مع أوبدون باستخدام مانع مضاد للبرولاكتين. وجدنا أن الكالسيوم في الدم وPRL و علامات الالتهاب قد زادت. انخفضت BMD بشكل كبير في الفئران المرضعة ، وزاد نشاط TRAP. لوحظ زيادة عدد Osteoclast وعلامات الامتصاص والتنشيط TRAP و MMP−9 و CTSK و CRP و RANK بشكل ملحوظ بعد العلاج ببروتين rPRL ، ولكن ليس في Osteoclast المعالجة بجسم مضاد لمستقبلات البرولاكتين و rPRL. تمت زيادة التعبير الجيني لـ TNF−α و IL−6 و MCP−1 ولكن ليس IL−1b بشكل كبير في Osteoclast مع علاج PRL مقارنةً Osteoclast غير المعالجة. وبالخلاصة، يمكن أن يؤدي المستوى العالي من PRL إلى تنشيط الخلايا الآكلة للعظم والتعبير عن السيتوكينات المنشطة للالتهابات التي تقلل كثافة المعادن بالعظام في فترة الرضاعة.

1. Introduction

Prolactin (PRL), a luteotropin hormone, is a protein secreted from the pituitary gland to stimulate and produce milk in the breast during the lactation period [1], especially when the progesterone is decreasing [2], [3]. PRL concentrations can be affected by nutritional and hormonal status [1]. PRL induces calcium release from bones as calcium storage to produce milk in mammary glands, and this may influence the bone strength [4]. The measurement of bone strength according to bone minerals like calcium and phosphate in bone tissue is called bone mineral density (BMD) [5]. Bone density measurement is used clinically to diagnose osteoporosis and fracture risk [6]. BMD has been found to be inversely correlated to PRL level [7], and bone turnover markers are associated with increased PRL concentrations [8]. It has been found that bone turnover especially bone resorption could be directly influenced by hyperprolactinemia, the high level of PRL decreases Osteoprotegerin (OPG) and increases Receptor activator of nuclear factor kappa-B (RANKL) from osteoblasts then induce osteoclast formation and activation [9], RANKL is one of TNF cytokines members and it is the ligand of RANK receptor on osteoclasts, the interaction of RANK-RANKL activates the osteoclast differentiation and activation that participates in bone resorption [10]. RANK is a

specific receptor expressed by osteoclasts and different immune cells such as DCs, macrophages, and microglia [11]. the RANK-RANKL interaction is a trigger for osteoclast differentiation which has a significant role in bone resorption [12].

PRL also plays a role as a cytokine activity for immune system activation through the nuclear factor kappa B (NF-κB) activation and this intracellular pathway could interfere with osteoclast differentiation. In addition, PRL could activate different immune cells which express the PRL receptor, leading to the secretion of inflammatory cytokines IL-6, TNF-alpha, MCP-1. These pro-inflammatory cytokines could have an essential role in osteoclast differentiation [13], [14]. The IL-6, and TNF-alpha have a direct effect on osteoclast differentiation and bone resorption *in vivo* [15], [16]. The high level of PRL hormone during lactation and its action on osteoclast activity and their differentiation is not clearly investigated. Tartrate resistance acid phosphatase (TRAP) is an enzymatic biomarker produced by osteoclasts [17]. TRAP has a functional activity in bone resorption [18]. TRAP has a dissolution action for bone minerals and degradation of organic bone matrix [19]. The aim of this study was to investigate the expression changes of osteoclasts biomarkers along with high levels of prolactin during lactation that could affect bone mineral density. Therefore, it was hypothesized that the high level of PRL during the lactation period could reduce bone mineral density through the direct effect on osteoclasts formation and activation.

2. Material and method

2.1. Serum Calcium and prolactin measurements.

To assess the effect of lactation on serum calcium and PRL level, the serum was collected in test tubes from female C57BL/6 mice during the period of lactation by direct heart puncture after general anesthesia. The blood clotting was performed for half an hour on ice; then the samples were centrifuged at 4°C for 15 minutes at 2,000g. Calcium concentration was measured following manufacturer instruction of calcium detection assay kit (Colorimetric) (Abcam cat# ab102505). The serum PRL concentration was measured using the prolactin mouse ELISA Kit (Abcam cat# ab100736).

2.2. Micro computed tomography.

To estimate bone mineral density, the femur, and 3rd lumbar vertebra were harvested from female lactating mice. The femurs and vertebrae of mice were fixed with 10% paraformaldehyde for 24hrs. The samples were scanned using a Caliper Micro-CT using PerkinElmer software at 90 Voltage Peak (VPK), 80μA, fold of view (FOV 5) for 3 minutes, tests were measured to an apparition for changing over the grayscale numbers to BMD units (mg/cc). This transformation is cultivated by utilizing a phantum of strong pitch implanted hydroxyapatite of realized densities to produce an adjustment line with fixations (0, 50, 200, 800, 1200 mg/cc). The image data of each sample was used to measure the bone microarchitecture values (BMA) using the developed software Analyze 12.0 (AnalyzeDirect.com).

2.3. Cell culture

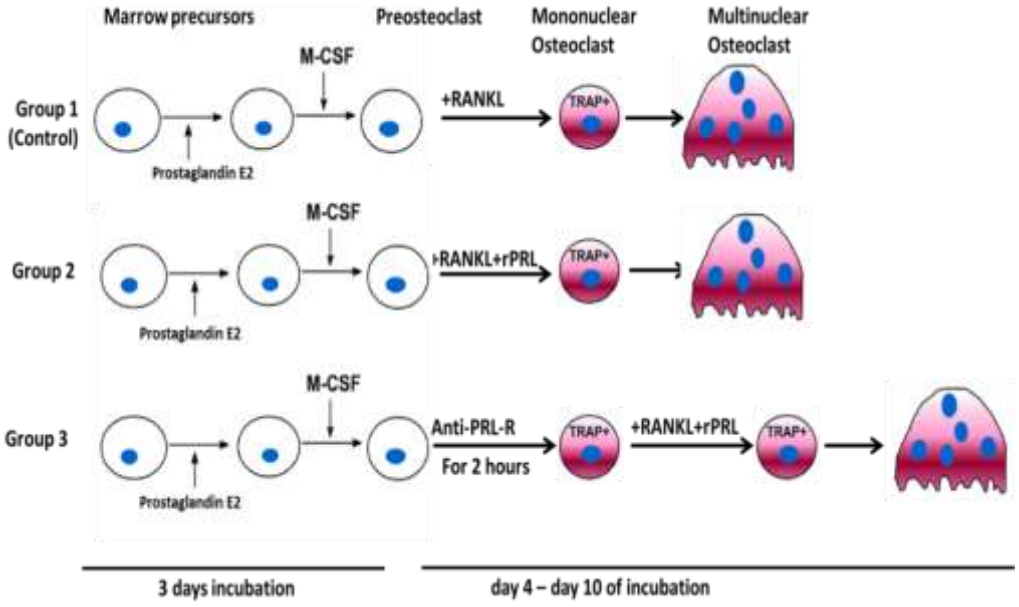
Osteoclasts were differentiated from the femur and tibia of six months female C57BL/6 mice. Bones were dissected and kept in Flow Cytometry Staining Buffer (FACS buffer) on ice, then they were crushed and filtered using a 70μm strainer into a new 50 ml conical tube for centrifugation twice at 200g, 5 min at 4°C for pellet formation [20]. The mononuclear cells were isolated from the suspended mixture by using the density gradient cell separation media (Histopaque-1077, Gibco). The number of isolated cells were cultured in basal media after adjusting to 2×10^5 cells/well into a 24-well plate with Minimal essential media (MEM)

without phenol red (Gibco) supplemented with, glutamate (1%), FCS (10%), 10,000 units/ml penicillin and streptomycin, 20 ng/ml recombinant Macrophage Colony Stimulating Factor M-CSF (R&D system), Prostaglandin E2 (Sigma-Aldrich) at 10^{-7}M final concentration, for three days. On day 4, in addition to previous supplements, the osteoclast induction media containing 20ng/ml of RANKL (R&D system) and/or without recombinant PRL protein at different concentrations (10ng/ml, 30ng/ml, and 50ng/ml) were added to cultured media for optimizing the effective concentration for osteoclast differentiation. The osteoclast induction media was changed daily to remove the remaining floating mononuclear cells.

Three groups of osteoclasts were differentiated, first untreated osteoclast culture as a control, the second osteoclast culture was treated with 50ng/ml of mouse recombinant prolactin protein (rPRL) (Abcam cat# ab243239), the third osteoclast culture was treated with mouse Anti-Prolactin Receptor antibody (Anti-PRL-R) (Abcam cat# ab2772) as a receptor blocker before 2 hours of 50ng/ml of mouse rPRL protein (see scheme 1).

Tartrate-resistant acid phosphatase stain was done to recognize the mature osteoclasts following the manufacturer instructions of TRAP Staining Kit (Kamiya Biomedical Company). The staining of tartrate-resistant acid phosphatase is used as a marker for osteoclasts detection.

The osteoclast resorption assay was performed by seeding osteoclasts on 24 well plate (Corning, cat# CLS3987-4EA) coated with hydroxyapatite. After 14 days, the wells were stained with Von Kossa stain which stains the hydroxyapatite, but the resorption area remains clear under the microscopic examination.



Scheme 1: The experimental design of osteoclast differentiation and treatment.

2.4. Western blotting

To quantify the protein expression of osteoclasts activation markers, the western blotting was used to detect the TRAP, RANK, C-Reactive Protein, Cathepsin K, MMP9 osteoclasts markers, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a normalizing protein from differentiated osteoclasts treated with 50ng/ml of mouse rPRL protein with or without anti-prolactin receptor antibody compared to untreated osteoclasts. The osteoclasts were differentiated from the femur and tibia using MCSF and RANKL as shown previously in the cell culture section above. The cell lysate was performed on ice using cell lysis buffer (Abcam

cat# ab152163) and the total protein concentration was measured using Nanodrop. The samples were denaturized at 95-100°C for 5 minutes and then 20µg of total protein was loaded to 10% of SDS-page under reducing conditions for 1 hour at 90 V. Then, a nitrocellulose membrane (ThermoFisher.com) was used for transferring proteins and the blocking of membrane with 0.05 skimmed milk was incubated for 60 minutes at 25°C then it was washed with washing buffer. The primary antibody followed by the secondary HRP antibody with 3 times washing steps between them were performed according target protein as shown in (Table 1) below. Western blot chemiluminescence reagents (Amersham Biosciences) were used to detect Immunoreactive proteins. Then, a Bio-Rad ChemDoc Touch imager was used to visualize the protein bands on the blot membrane. The GAPDH and Beta-Actin as housekeeping proteins were used as normalizing proteins for the target proteins. The experiment was repeated 5 times with 4 replicates for each sample loaded.

Table 1: shows the target protein, molecular weight, primary antibody, and secondary antibody.

Target protein	Molecular weight	Primary antibody	Secondary antibody
TRAP	60kDa	Rabbit anti-Mouse TRAP/CD40L antibody (ab2391)	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
RANK	97 kDa	Rabbit anti-Mouse RANK Polyclonal Antibody (MBS178245)	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
C-Reactive Protein	26kDa	Rabbit anti-Mouse -C Reactive Protein antibody (ab207756)	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
Cathepsin K	37 kDa	Rabbit Anti-Mouse Cathepsin K antibody (ab19027)	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
MMP9	105 kDa	Rabbit Anti-Mouse MMP9 antibody (ab38898)	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
GAPDH	36 kDa	Rabbit Anti-mouse GAPDH antibody (ab181602)	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
β-Actin	45 kDa	Rabbit Anti-mouse Anti-beta Actin antibody (ab8227)	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
IL-6	23 kDa	Rabbit Anti-mouse Recombinant Anti-IL-6 antibody [EPR23819-11] (ab259341)	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
TNF-α	26 kDa	Rabbit polyclonal Anti-TNF alpha antibody [EPR20972] ab215188	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
IL-1-beta	30 kDa	Rabbit monoclonal Anti-IL-1 beta antibody [EPR23851-127] ab254360	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)
MCP-1	18kDa	Rabbit polyclonal Anti-MCP1 antibody (ab7202)	Goat Anti-Rabbit IgG H&L (HRP) (ab205718)

2.5. qPCR

Extraction of total RNA was done by Trizol Reagent (Invitrogen, Paisley, UK) from cultured osteoclast treated with rPRL protein with or without anti-prolactin receptor. RevertAid H Minus Kit (Thermo scientific K1632) for RT-PCR (Invitrogen) was utilized to synthesize the First strand cDNA was from total RNA. The RT-qPCR technique using SYBR

Green I dye (SensiMixTM SYBR Kit, Cat.QT605) was used to quantify gene expression. The qPCR reactions were performed using 20µl total volume strip tubes and caps. Each tube contains 3µl of cDNA (previously diluted 1:4 in dH2O), 10µl SYBRGreen (containing MgCl2) and 2µl of diluted forward primer (5µM), 2µl of diluted reverse primer (5µM) for genes of interest (1µl of stock primer (100µM) in 19µl dH2O for each forward and reverse primer) and made up to 20µl by adding 3µl dH2O. In parallel, water rather than cDNA template was used as a negative control (NTC). The gene expression was quantified and normalised to GAPDH expression. The samples of 20µl total volume qPCR reaction were run on the Corbett: Rotor-GeneTM 6000 machines and software using the RT-qPCR reaction program. The temperature cycling conditions and primers sequences are shown in Table 2.

Table 2: primers used to detect the qPCR product.

Name of Primer	Sequence of Primer
TRAP	For: 5'-AGCAGCTCCCTAGAAGATGGAT-3' Rev: 5'- TTCTGGCGATCTCTTTGGCA-3'
C-reactive protein	For: 5'-GCGGAAAAGTCTGCACAAGG-3' Rev: 5'-GCTCTAGTGCTGAGGACCAC-3'
RANK	For: 5'-CACTCCTACCTCCGACAGTG-3' Rev: 5'-ACCGTATCCTTGTTGAGCTGC-3'
Cathepsin K	For: 5'-CTAGCACCCTTAGTCTTCCGC-3' Rev: 5'-GAGAGGCCTCCAGGTTATGG-3'
MMP-9	For: 5'-TGGGTGTACACAGGCAAGAC-3' Rev: 5'-TGGAAACTCACACGCCAGAA-3'
GAPDH	For: 5'-CCCTTAAGAGGGATGCTGCC-3' Rev: 5'-ACTGTGCCGTTGAATTTGCC-3'.

2.6. Measurement of inflammatory cytokines

The Murine TNF-α ELISA development kit (PeproTech 900-TM54) was used to quantify the level of TNF-alpha in mice serum. The quantitative measurement of natural murine TNF-α was performed in a sandwich ELISA format within the range of 10–2500pg/ml.

The murine IL-6 ELISA development kit (PeproTech, 900-TM50) was used to quantify the level of IL-6 in mice serum. The quantitative measurement of natural murine IL-6 was performed in a sandwich ELISA format within the range of 32-4000pg/ml.
The murine MCP-1 ELISA development kit (Abcam cat# ab100721) was used to quantify the level of MCP-1 in mouse serum. The quantitative measurement of natural murine MCP-1 was performed in a sandwich ELISA format within the range of 2.74pg/ml - 2000pg/ml.
The Mouse IL-1 beta ELISA Kit (Abcam cat# ab197742) was used to quantify the level of IL-1 beta in mouse serum. The quantitative measurement of natural murine IL-1 beta was performed in a sandwich ELISA format within the range of 1.56pg/ml - 100pg/ml.

2.7. Immunohistochemistry

The femur bones of subjected mice in this study were dissected, and cleaned from the soft tissue, then they were fixed with 10% paraformaldehyde for 3 days. After fixation, the decalcification was done by 20% of EDTA for 10 days. Then the decalcificated samples were embedded in paraffin blogs for detecting TRAP activity of osteoclasts by immunohistochemistry. The paraffin sections were deparaffinized and rehydrated using Xylene,100% ethanol, 95% ethanol. Then the slides were washed 2 x 5 minutes in TBS plus 0.025% Triton X-100 with gentle agitation. Then, the blocking step with 10% normal serum with 1% BSA in TBS for 2 hours at room temperature. Slides were drained for a few seconds and wiped around the sections with tissue paper. TRAP antibody (Biorbyt cat# orb36683), a purified rabbit polyclonal antibody was applied as primary antibody diluted (1:20) in TBS

with 1% BSA, and incubate overnight at 4°C. Next day, the slides were rinsed 2 x 5min TBS 0.025% Triton with gentle agitation. The slides were incubated in 0.3% H_2O_2 in TBS for 15 min. Then, the Goat Anti-Rabbit IgG H&L (HRP) (Abcam cat# ab205718) was applied as a secondary antibody and diluted in TBS with 1% BSA, and incubated for 1 hour at room temperature, then, it was rinsed 2 x 5min TBS 0.025% Triton with gentle agitation. Chromogen (DAB) was developed for 10 min at room temperature and then, rinsed in running tap water for 5 min, the Counterstain H&E was applied then dehydrated for microscopic examination.

2.8. Statistical analysis

Data were presented as means ± SEM and analysed by unpaired *t*-test or one-way ANOVA where appropriate using Prism Pad 8. The P-value of *p<0.05 was deemed as significant.

3. Result and Discussion

The serum Calcium and prolactin levels increase during the lactation period.

The serum calcium and PRL levels in six months old lactating female C57BL/6 and female mice (control) were measured to study the changes in BMD. The serum measurements showed that the calcium level and PRL level were significantly increased in those female mice during the lactation period (7 days postnatal) (Figure 1). The lactating mice had higher PRL and calcium concentrations in serum (p<0.0001).

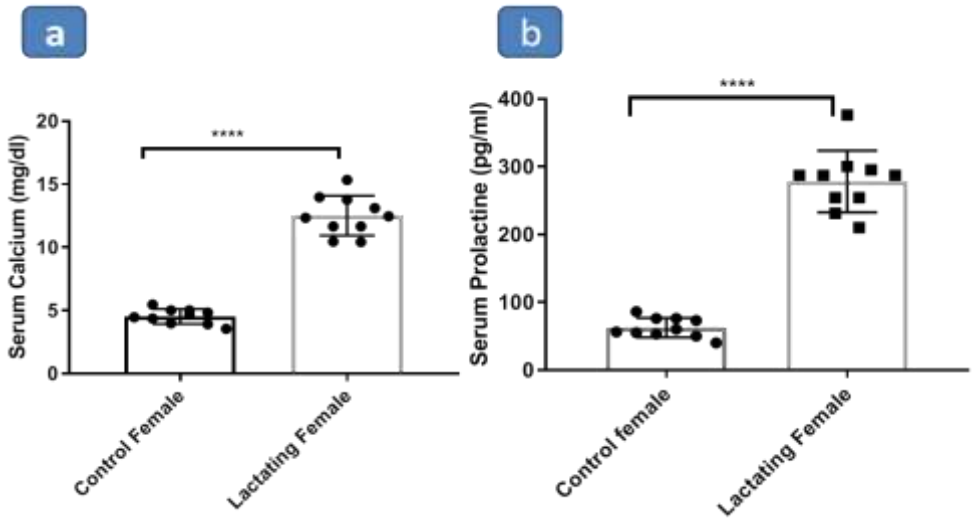


Figure 1: The serum calcium and PRL levels were measured in lactating and control mice at six months old n=10 in each group. Serum calcium concentration (a), Serum PRL hormone concentration (b). The results are expressed as means ± SEM (unpaired t-test **** p<0.0001) measured in triplicate.

High level of prolactin hormone combined with BMD reduction.

BMD was measured in female mice at lactation period when they have a high PRL concentration (Figure 1). BMD of the femur and third lumbar vertebra was assessed using micro-computed tomography technique. Bone microarchitecture imaging showed that in both femur and 3rd lumbar vertebra have a significant reduction in bone density in lactating female mice compared to control mice (Figure 2). Bone parameters were reduced in lactating mice in both the femur and vertebra. The compact bone was affected by high PRL concentration, the whole bone mineral density, bone mean BMD, cortex mean BMD were significantly decreased in lactating mice compared to control mice (Figure 3 a, b, and c). The microenvironment of cancellous bone was significantly affected during the lactation or high level of PRL hormone. The trabecular BMD, bone volume fraction (BV/TV), trabecular

thickness, and trabecular number were significantly decreased in female mice during lactation compared to control mice (Figure 3 d, e, f, and g).

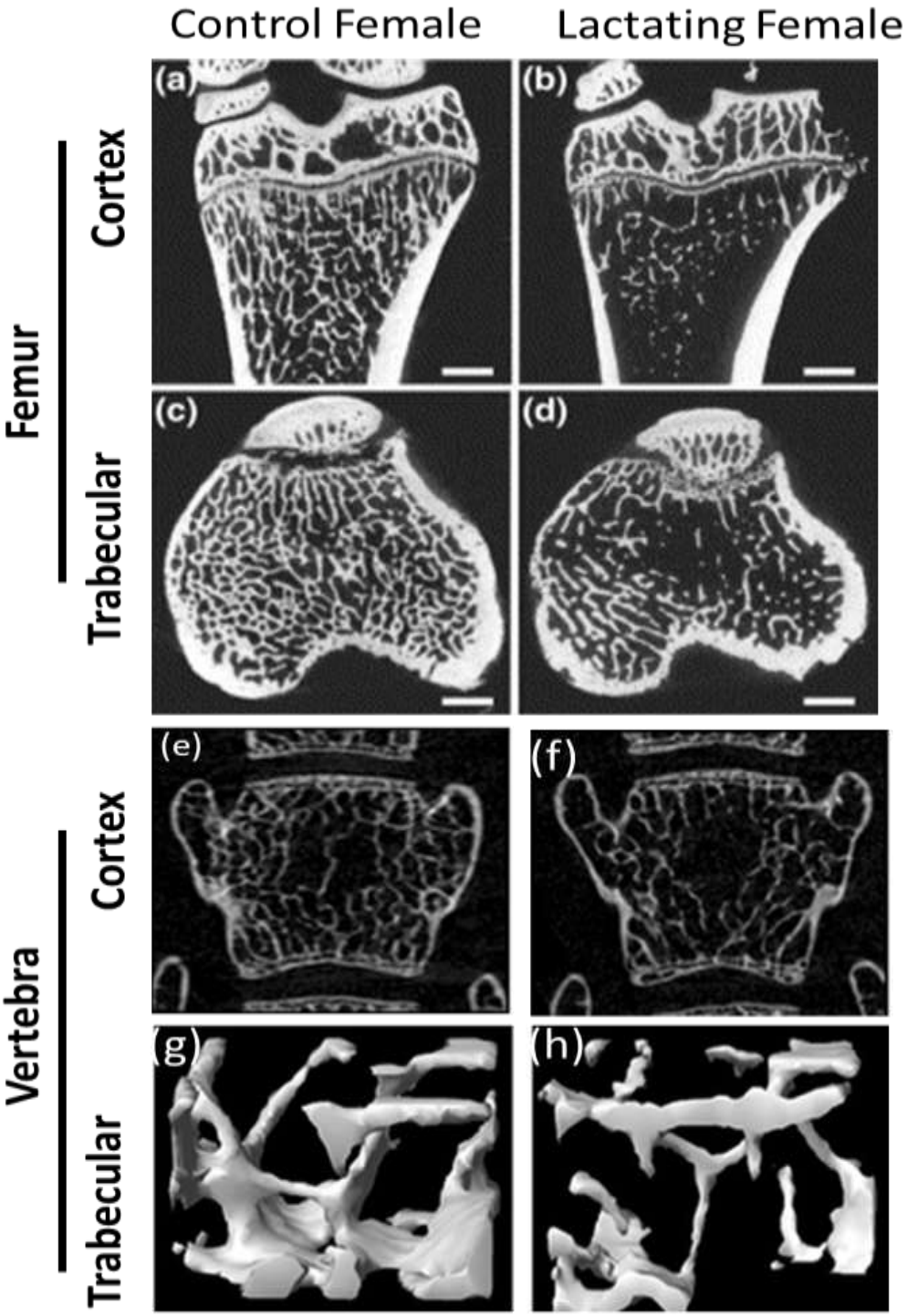


Figure 2: A representative pictures of μ-CT imaging of the femur and 3rd lumbar vertebra of female mice during the lactating period compared to control mice (n=10 for each group).

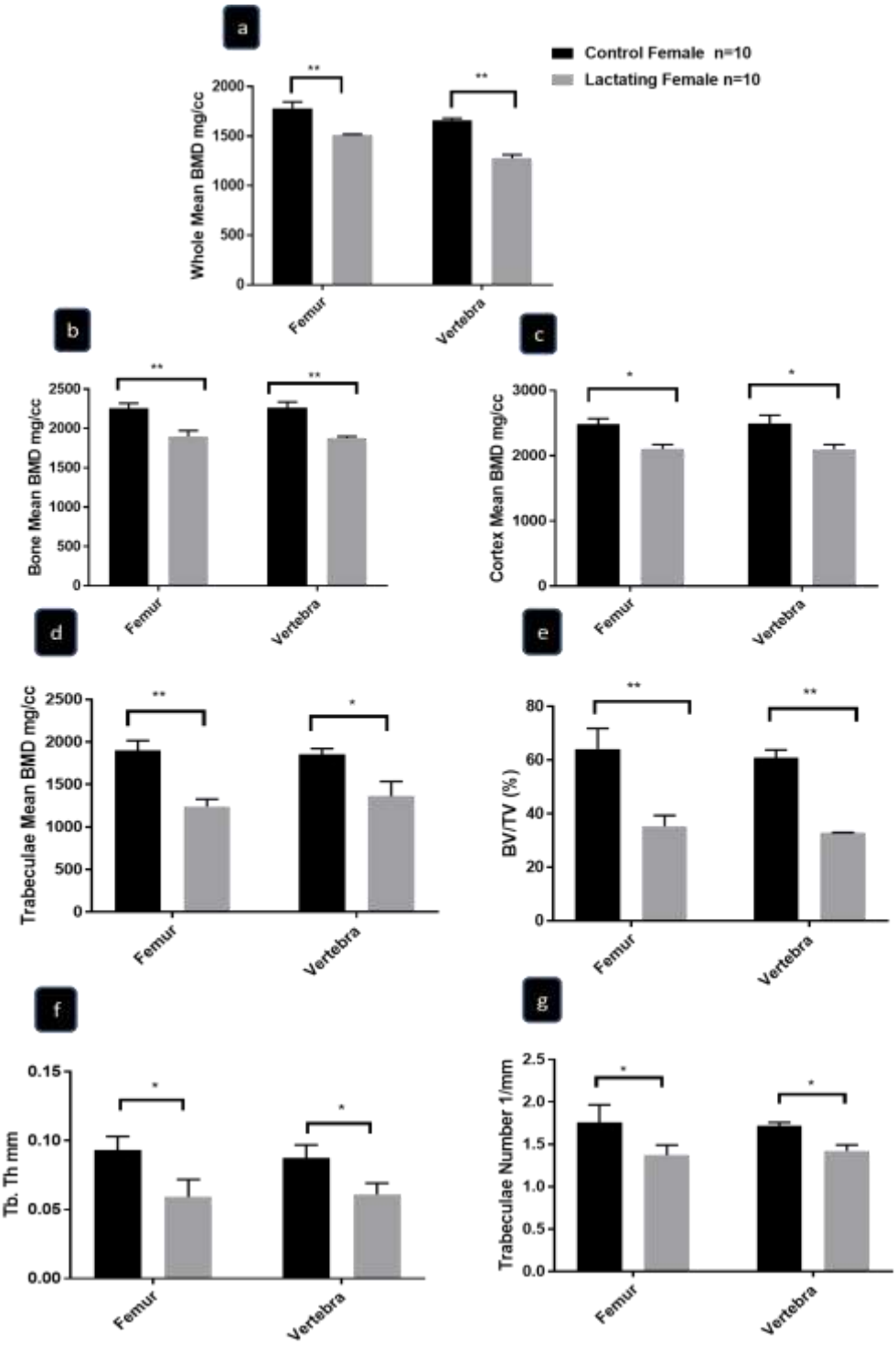


Figure 3: bone mineral density measurement of the femur and 3rd lumbar vertebra of control and lactating female mice. (a) Whole bone mineral density, (b) bone mean density, (c) Cortex mean density, (d) Trabecular Mean density, (e) BV/TV%, (f) Trabecular thickness, (g) Trabecular number. The results are showed as means of ± SEM (t-test **P* < 0.05; ***P* < 0.01).

Prolactin hormone activates osteoclast formation.

In vitro experiments were performed to assess the relationship between bone resorption and osteoclast activation after treating the differentiated osteoclasts with recombinant PRL protein. The osteoclast cultures were treated with different concentrations to detect the dose-response of rPRL hormone (10ng/ml, 30ng/ml, and 50ng/ml) and osteoclast differentiation.

Results show that a 50ng/ml dose of rPRL was most effective to induce osteoclast differentiation, as shown in Figure (4-d) there are huge mature multinucleated osteoclasts that could refer to the effect of PRL high concentration (50ng/ml), therefore it was used for osteoclast stimulation (Figure 4 a-d).

Osteoclasts were differentiated from monocytes isolated from the bone marrow of female wildtype mice femur and the cultures were treated with 50ng/ml of rPRL with or without anti-PRL-R. The differentiated osteoclasts were stained with TRACP staining followed manufacturer instructions to detect the mature differentiated osteoclasts. The result showed that the number of TRAP positive osteoclasts formation was higher in osteoclast culture treated with PRL compared to untreated osteoclast culture, however, the osteoclasts formation treated with anti-PRL-R and with rPRL hormone was comparable to untreated osteoclast culture (Figure 4 e, f, and g). The osteoclast bone resorption area of osteoclast culture treated with rPRL was higher than the untreated osteoclast culture, but the osteoclast treated with rPRL and anti-PRL-R was the same as untreated osteoclasts (Figure 4 h, i, and j). This could elucidate the effect of PRL hormone on osteoclast activation.

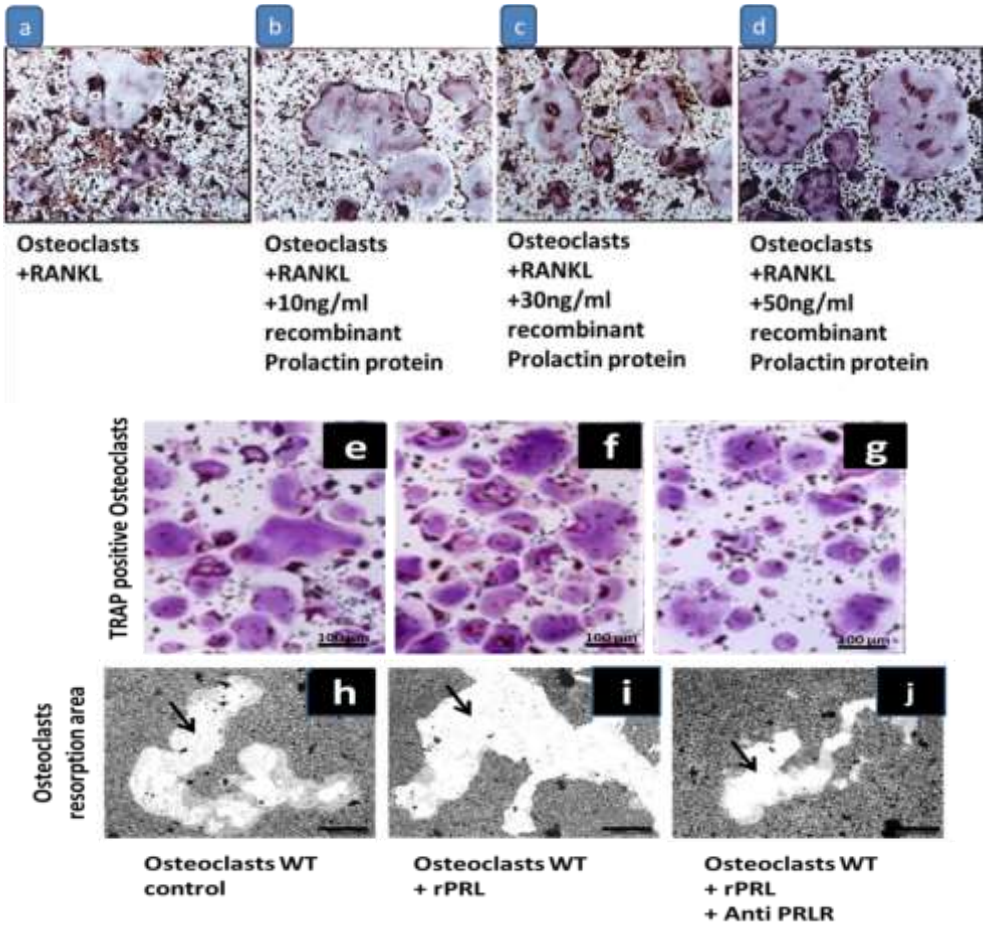


Figure 4: **Osteoclast activity in response to rPRL stimulation**: Detection the effective concentration of rPRL protein (a-d), it was used different concentrations of rPRL hormone (10ng/ml, 30ng/ml and 50ng/ml) to find the effective concentration for osteoclasts (OC) differentiation. Differentiated TRAP positive osteoclast treated with or without rPRL or/and anti-PRL-R (e-g), the differentiated OC were stained by TRACP staining to detect the mature OC. Bone resorption of treated or untreated osteoclast, arrows refer to osteoclast resorption area (h-j).

Prolactin hormone induces osteoclast makers expression

Different markers expression for osteoclast activation could be affected by PRL hormone. The protein expression of Tartrate-resistant acid phosphatase (TRAP), C-reactive protein, Cathepsin K (CTS-K), receptor activator nuclear kappa B (Rank), Matrix metallopeptidase 9 (MMP-9) were significantly increased in osteoclast after treating them with rPRL protein (Figure 5 lanes 2) compared to untreated osteoclast (Figure 5 lanes 1). However, the protein expression of osteoclasts markers treated with anti-prolactin receptor and then rPRL protein were the same as untreated osteoclast (Figure 5 lanes 3). The densitometry analysis was done to quantify the bands density for osteoclasts markers before and after treatment. This could indicate that PRL hormone could induce osteoclast activation. The GAPDH and Beta actin were used as normalizing protein as housekeeping proteins to compare the loaded target proteins.

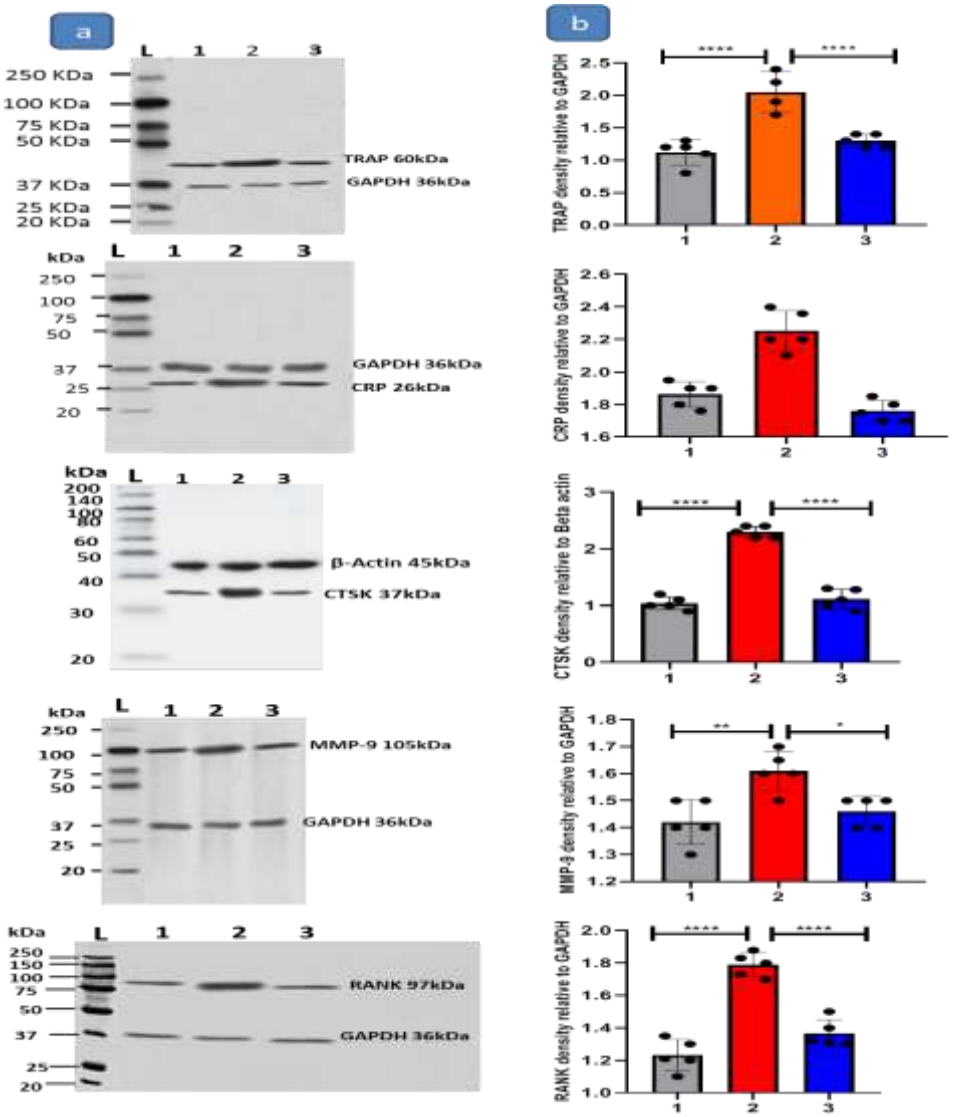


Figure 5: A representative images for Osteoclast markers protein expression by western blot analyses, (a) lane 1, untreated control osteoclasts. The expression of osteoclasts markers were increased in osteoclasts treated with rPRL protein (Lane 2). However, the osteoclasts treated with anti-prolactin receptor antibody and then rPRL protein, the osteoclast markers were comparable to the control (Lane 3). The density of detected bands were analyzed using densitometry (b) to express the differences in WB blotting for each group. The data represented as means of ± SEM (t-test **P* < 0.05; ***P* < 0.01; **** p<0.0001 where n=3 of experiments).

3.5. Prolactin increases inflammatory gene expression in osteoclasts.

The inflammatory cytokine gene expressions were measured in differentiated osteoclasts treated with or without rPRL hormone. The results showed that there are significant increases in gene expressions of *TNF-α, IL-6*, and *MCP-1* but not *IL-1β* in osteoclasts treated with rPRL protein compared to control osteoclasts culture (Figure 6). The inflammatory cytokines expression in osteoclasts treated with rPRL protein and PRL receptor blocker were comparable to the expression in untreated osteoclast (Figure 6).

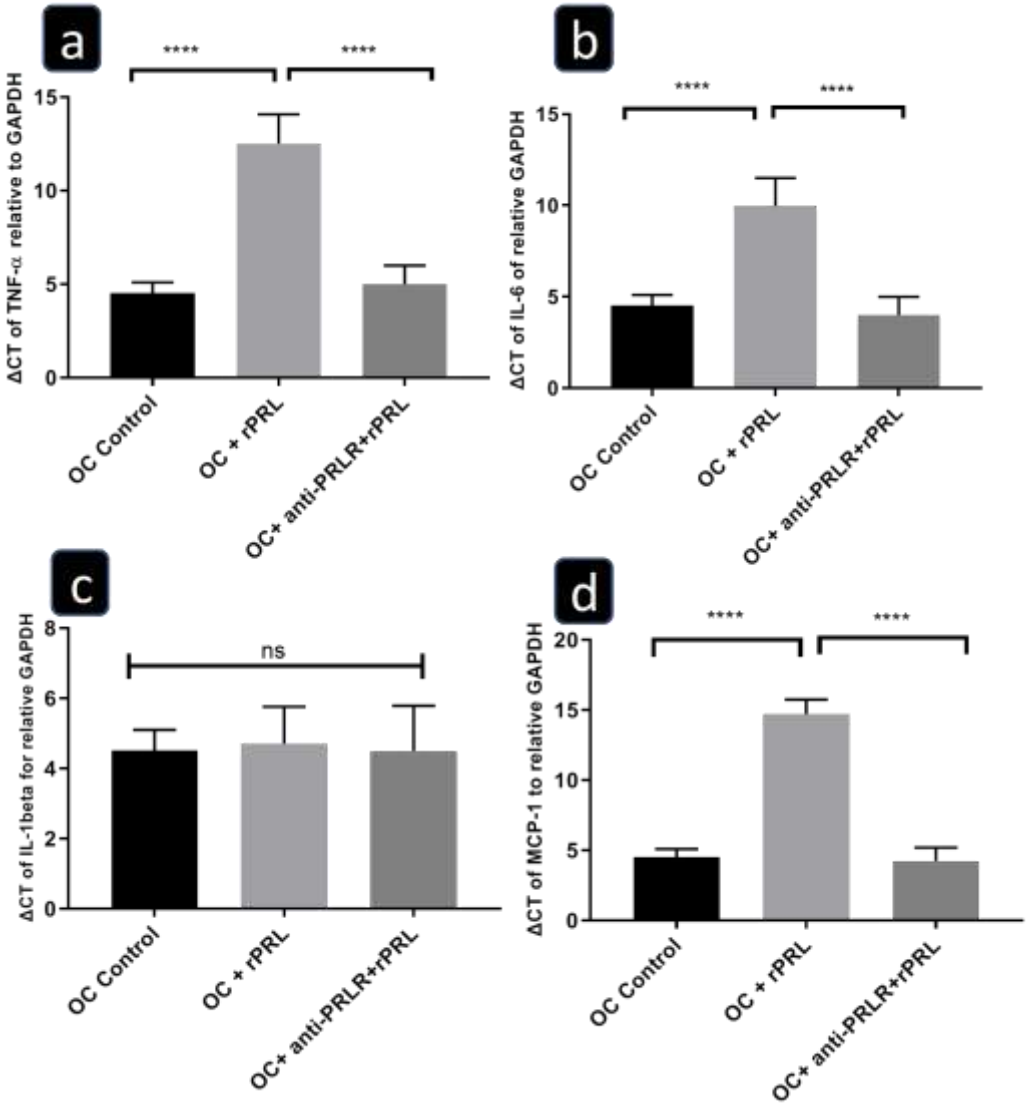


Figure 6: Inflammatory cytokines gene expression in differentiated osteoclast treated with or without rPRL protein or anti-prolactin receptor antibody. (a) TNF-α gene expression, (b*) IL-6*, (c) *IL-1β*, (d) *MCP-1*. The statistics as means of ± SEM (t-test ****$P < 0.0001$ and ns= no significant differences).

Serum Inflammatory markers change in mice during lactation period.

The inflammatory cytokine concentrations were measured in the serum of control mice and lactating mice to validate the in vitro gene expression of inflammatory cytokines. The results showed that there are significant increases in serum concentration levels of TNF-α, IL-6, and MCP-1 but not IL-1β in lactating mice compared to control mice (Figure 7). These results could confirm the significant changes in inflammatory gene expression in (Figure 6)

and may give the understanding that the PRL could be a regulator for inflammatory cytokines gene expression.

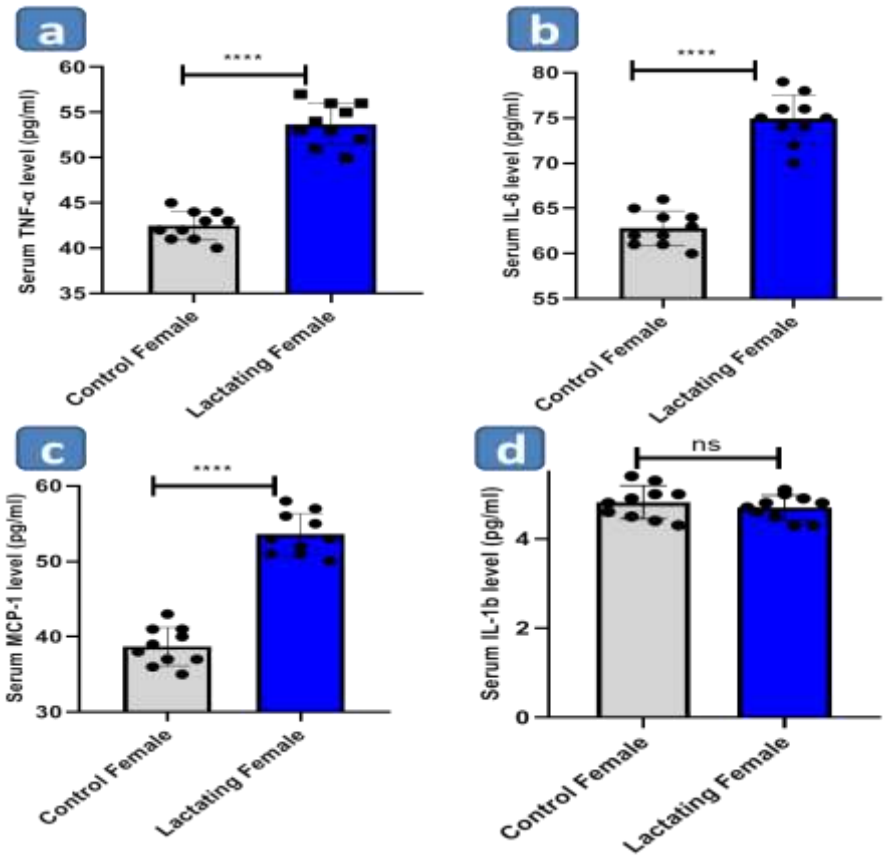


Figure 7: Serum inflammatory cytokine markers measurements in lactating mice: The inflammatory markers were evaluated in serum mice subjected to this study for estimating the effect of PRL high levels on TNF- α, IL-6, MCP-1, and IL-b1 during lactation period. (a) the serum level TNF- α; (b) the serum level of IL-6; (c) the serum level of MCP-1; (d) the serum level of IL-1b. The statistics as means of ± SEM (t-test ****$P < 0.0001$ and ns= no significant differences) where n=10.

In *vivo* of bone microarchitecture change in lactating mice

The immunohistochemistry for TRAP regarding to osteoclast activity in mice bones was detected in paraffin embedded bone after bone decalcification with EDTA. The result revealed that the TRAP was detected in bone section in lactating mice was more than in normal mice at the same age (six months) (Figure 8 A&B). In addition, the H&E staining was completed to depict the microarchitecture environment of bone mice. The H&E staining showed that the trabeculae in lactating mice was less than in normal mice (Figure 8 C&D).

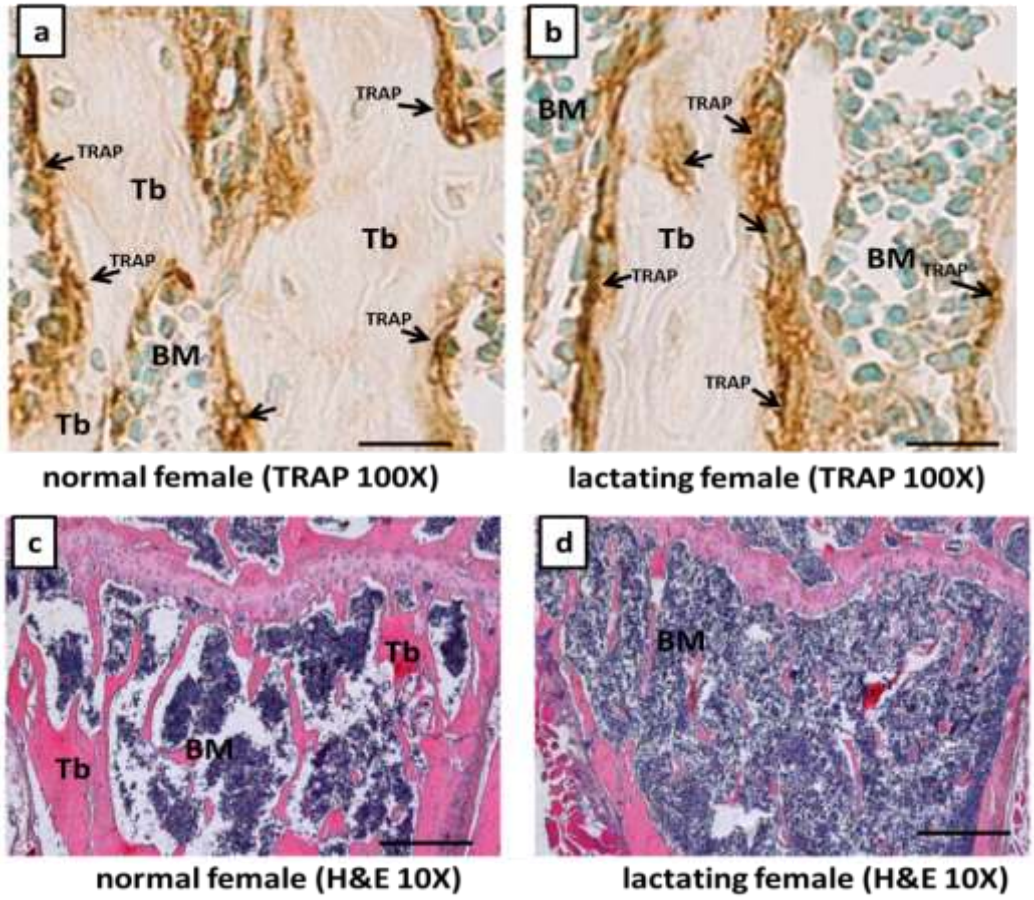


Figure 8: histological analysis observing the bone microarchitecture changes: the immunohistochemistry detection for TRAP activity in the bone of normal and lactation mice (a&b) arrows refer to $TRAP^{+}$ OC activity. The H&E staining shows the trabeculae changing between normal and lactation mice (c&d).

Prolactin enhances inflammatory cytokines production in osteoclasts.

The inflammatory cytokines levels produced by osteoclasts which treated with or without rPRL were measured by western blotting to determine the IL-6, TNF-α, IL-1 beta, and MCP-1 as inflammatory cytokines. The results showed that protein expression of the inflammatory cytokines IL-6, TNF-α, IL-1 beta, and MCP-1 were significantly increased in osteoclast treated with rPRL (Figure 9 lane 2) compared to untreated osteoclast, however, there were no differences between the blocked osteoclasts cultures and untreated osteoclasts in the expression of the inflammatory cytokine (Figures 9 lane 1 and 3).

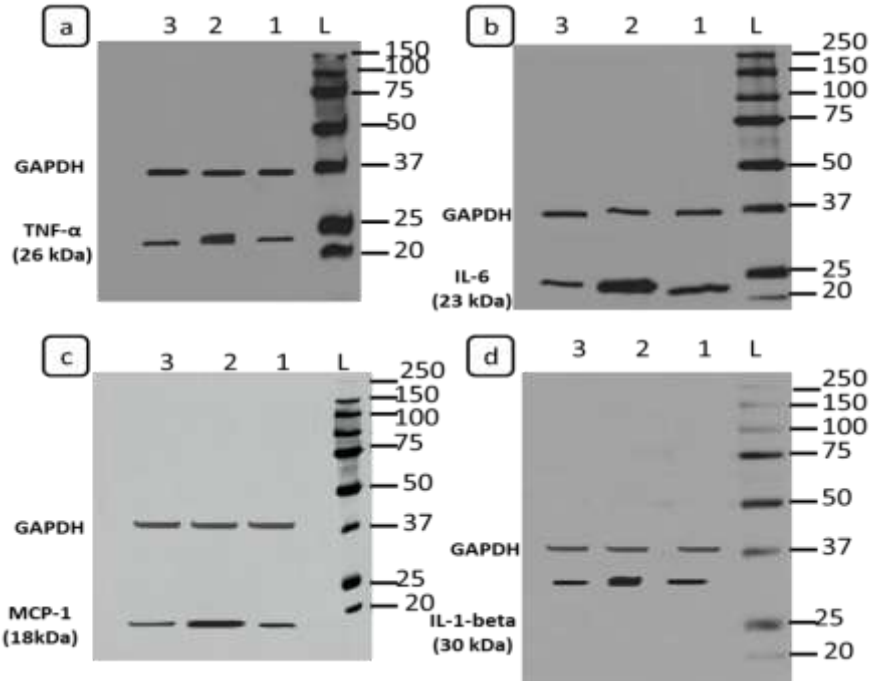


Figure 9: the expression of the inflammatory protein from differentiated mouse osteoclasts, lane 1 indicated cell lysate from untreated osteoclasts, lane 2 refers to cell lysate of osteoclast treated with rPRL, lane 3 refers to cell lysate of osteoclast treated with anti-PRL Ab and then with rPRL. (a) the expression of TNF-α, (b) the expression of IL-6, (c) the expression of MCP-1, and (d) the expression of IL-1 beta.

PRL is a hormone produced and secreted from the pituitary gland, it has an essential role during pregnancy and lactation period [21]. PRL has a fundamental action to activate mammary glands to produce and secret milk [22]. Calcium releasing from bone to the bloodstream or milk could be regulated by PRL hormone [23], [24]. Serum calcium concentration was significantly increased and associated with high concentrations of PRL in lactating females [25]. Our data showed that serum calcium concentration was significantly increased in lactating female mice compared to control mice, suggesting that the high PRL may be related to releases of calcium from bones resulting in BMD disturbances. Hormonal changes like low estrogen and high parathyroid hormones induce deleterious alterations in bone turnover and bone loss [26]. In humans, it was found that there are about 20-30% of bone loss in lactating women [27], also the bone cortex density was significantly reduced over the lactation [28]. Our results presented that lactating female mice, there were significant losses in (BV/TV%), the percentage of minerals to bone volume of the interested region (Figure 3 e). The bone volume fraction, thickness, and number of trabeculae of femur and vertebrae was lower in lactating mice compared to control (Figure 3 e, g). The internal trabecular microarchitecture of femur and vertebra in lactating mice was significantly decreased in comparison with control mice, that may participate in a reduction in trabecular bone mineral density (Figures 2 and Fig 3 d). In addition, the bone mean mineral density, cortex bone mineral density, and whole bone mineral density were significantly decreased in lactating mice compared to non-lactating female mice (Figure 2 and Figure 3 a, b, c). This led to the speculation that BMD could be reduced due to high lactation hormones concentrations.

High activity of osteoclast induced a reduction in BMD [29], [30]. The effect of PRL hormone on osteoclast activity and their differentiation is not fully understood. It was shown that there is a direct effect of PRL hormone on RANKL expression which induces osteoclast differentiation and activation [9], our findings showed that the number of osteoclasts and their

bone resorption activity was increased after treating them with rPRL compared to untreated osteoclasts (Figure 4 e, f and h, i), providing an explanation for the effect of PRL and osteoclast behavior. However, the number and osteoclast resorption has been not changed in osteoclast cultures treated with PRL receptor blocker antibody and rPRL (Figure 4 g and j). This leads to a speculation that PRL protein via its receptor could induce a signal cascade into osteoclasts to increase their activity.

There are different osteoclast biomarkers which could give an indication of their activity. Receptor activator of nuclear factor-kappa B (RANK), a specific receptor for receptor activator of nuclear factor-kappa B ligand (RANKL) that contribute in osteoclastogenesis [31]. During lactation period, the RANK expression was increased in epithelial cells of the mammary gland [32], also, the RANKL expression are increased during the lactation period and hormonal level changes [33]. Our data represent a high expression of RANK protein in differentiated osteoclasts stimulated with rPRL hormone, suggesting that increases in osteoclast differentiation rate that could lead to increase in the resorption activity of osteoclast. Tartrate resistance acid phosphatase (TRAP) is a specific enzyme highly expressed by osteoclasts, activated macrophages, neurons, and by the porcine endometrium during pregnancy [34]. It was shown that TRAP was highly expressed in murine osteoclast during the lactation period [35]. Our data showed an increase in the expression of TRAP protein in differentiated osteoclasts treated with rPRL, thus indicating the high activity of osteoclasts during the lactation period which may decrease bone mineral density.

Cathepsin K (CTS-K) is a lysosomal cysteine protease contributes in bone resorption, and secreted mainly by mature osteoclasts [36]. The CTS-K expression was significantly increased in murine osteoclast during lactation [35], in our *in vitro* experiment the CTS-K was significantly increased in treated osteoclast compared to untreated, therefore bone resorption increases in the bones of lactating mice. Matrix metallopeptidase 9 (MMP-9) it is a type IV collagenase that dissolve the extracellular matrix [37]. MMP-9 was found to be expressed by osteoclast and it has an important role in bone resorption by lysing and degradation collagen [38]. Also, it was found that the high expression of CTS-K could cleave and activate the MMP-9 to contributing to bone resorption [39]. Our data shows MMP-9 protein expression was significantly increased in differentiated osteoclast stimulated with rPRL, providing further support that PRL leads in osteoclast activation, bone resoprtion and ultimately bone mineral density reduction. C-reactive protein is a protein secreted to the blood stream resulting to inflammation. C-reactive protein affect the osteoclast differentiation by inducing RANKL expression increase from monocytes, and this could induce bone resorption [40], [41]. It was shown that the C-reactive protein increase was associated with the stress conditions of heavy lactation [42]. Our findings show that the protein expression of C-reactive protein was increased in the osteoclast culture treated with rPRL compared to the control. We conclude that PRL may induce a low-grade inflammatory response. The inflammatory cytokine gene expressions by osteoclasts were measured to investigate whether the rPRL may affect the gene expression of *TNF-α , IL-6, MCP-1* and *IL-1beta*. Since osteoclasts are derived from the macrophage/monocytes lineage, the PRLR activation could up-regulate the *TNF-α* gene expression[43]. Also, it was shown that *IL-6* gene expression was up regulated in lactating cow [44]. The MCP-1 was significantly expressed in macrophages treated with PRL [45]. Our data showed that gene expression of some inflammatory markers like *TNF-α, IL-6* and *MCP-1* were significantly up-regulated in osteoclasts stimulated with rPRL, but not IL-1beta. These results suggesting that inflammatory cytokines are increased during the lactation period and this may lead to osteoclast activation and deterioration of BMD. As the gene expression of inflammatory cytokines were up regulated in differentiated osteoclasts treated

with rPRL, therefore it was important to measure the protein expression of inflammatory cytokines in stimulated differentiated osteoclasts, in addition to the serum levels of inflammatory cytokines in the live mice during the lactating period. In vitro, the expression of *TNF-α, IL-6* and *MCP-1* and IL-1beta were increased after osteoclast stimulation compared to untreated osteoclast, however, in vivo the *TNF-α, IL-6* and *MCP-1* (not IL-1beta) were higher during lactating period in mice. It is not shown the effect of PRL on serum inflammatory cytokines levels, but it is suggesting that a high level of PRL during lactating period could affect different immune cells like macrophages , TH17, Th1 and other [46], [47], the inflammatory cytokines expression and regulation has been shown to be regulated by PRL hormone [43], [48] . The important point is that the BMD could be reduced resulting to high activity of osteoclast. Our results in paraffin embedded bone sections using immunohistochemistry showed that the TRAP activity by osteoclasts was higher in lactating mice than normal mice. It was shown by Qing et al (2012) that the TRAP activity was elevated in lactation mice [35]. In addition, the H&E staining of bone sections showed that the trabeculae lactation mice were reduced when compared to non-lactating mice. These results could elucidate the BMD reduction as the osteoclasts TRAP activity was higher in lactating mice than normal mice maybe because the high concentration of PRL. Therefore, high concentrations of TRAP lead to high osteoclasts activity and lowering of BMD.

In conclusion, the *in vitro* work showed that PRL could increase gene expression of inflammatory cytokines (*TNF-α, IL-6, MCP-1*) in murine differentiated osteoclasts. Also, *in vitro* PRL stimulation showed a significant osteoclast differentiation and activation by increase the number of osteoclast and RANK protein expression. In addition, the osteoclast resorption activity was increased after PRL stimulation, and this is represented by increasing the osteoclast resorption activity markers like TRAP, MMP-9, CRP and CTS-K. These results are in agreement with the *in vivo* experiment of serum cytokines level elevation and micro-computed tomography that showed a significant reduction in bone microarchitecture of BMD in mice during lactation period. The osteoclastic activity was higher than in non-lactating mice (TRAP activity) which reflected the differences in BMD between the lactating and non-lactating mice. These findings provide further evidence that PRL contributes to the pro-inflammatory cytokines, osteoclastic activity and BMD during lactation.

4. Acknowledgements

This manuscript contains a part of Z. Saeed's research work. We thank Al-Furat Al-Awsat Technical University for its financial support (ZS). We are grateful to Dr. Baleegh A Kadhim and Dr. R. Kheder, Dr. Hobkirk James (Hull University, UK), and Dr. Cordula M. Stover (Leicester University, UK) for useful discussions. Dr. Mohenned A. Alsaadawi had a potential role in the added experiment as reviewers requested. We appreciate the contribution of the Al-Qadisiyah University (Division of Biological Sciences) regarding the legal framework of the undertaken work and the Preclinical Research Facility of the Al-Qadisiyah University regarding the husbandry of the experimental mice.

5. Authors' contribution statement

Zeayd Saeed, Baleegh A Kadhim and Ali Mosa Rashid Al-Yasari designed and carried out the study. Sura Abdulhussein and Ramiar K. Kheder oversaw the study and Zeayd Saeed wrote the paper, Ramiar Kheder and Hobkirk James (Hull University, UK) had the role in substantial discussion of this work and English language revision. Mohenned A. Alsaadawi had participated in the added experiment as reviewers requested and revised the paper. All authors approved the content.

6. Disclosure and conflict of interest

The authors declare that they have no conflicts of interest."

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