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ORIGINAL ARTICLE

Expression of prolactin receptors in the duodenum, kidneys and skeletal system during physiological and sulpiride-induced hyperprolactinaemia

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Abstract

Introduction and aim Hyperprolactinaemia in pregnancy leads to mild and reversible changes in the maternal skeletal system, and medicamentous hyperprolactinemia causes more detrimental effects. We conducted an experimental study to evaluate differences between *Prlr* gene expression in the duodenum, vertebrae and kidneys during physiological and medicamentous hyperprolactinaemia, which could influence calcium homeostasis.

Methods Experimental animals (18 weeks old, Wistar female rats) were divided as follows: group P (nine rats that were 3 weeks pregnant), group M (ten rats that were intramuscularly administrated sulpiride (10 mg/kg) twice daily for 3 weeks), and the control group (C, ten age-matched nulliparous rats, 18-week-old). Laboratory investigations included measurements of serum ionized calcium, phosphorus, urinary calcium and phosphorus excretion, osteocalcin (OC), serum procollagen type 1 N-terminal propeptide (P1NP), vitamin D, parathyroid hormone (PTH) and prolactin (PRL). Relative quantification of gene expression for prolactin receptors in the duodenum, vertebrae and kidneys was determined using real-time PCR.

Results Expression of the *Prlr* gene was significantly higher in the duodenum ($p < 0.001$) and lower in vertebrae ($p < 0.001$) and kidneys ($p < 0.01$) in rats with physiological hyperprolactinaemia (PHP) than in the control group. Significantly lower *Prlr* expression in the duodenum was verified ($p < 0.001$), along with increased *Prlr* gene expression in vertebrae ($p < 0.001$) and kidneys ($p < 0.01$), in rats with medicamentous hyperprolactinaemia (MHP) than in the C group.

Conclusions Downregulation of *Prlr* gene expression in the duodenum may explain the diminished intestinal calcium absorption in medicamentous hyperprolactinaemia. Prolactin takes calcium from the skeletal system following increased *Prlr* gene expression in the vertebrae to maintain calcium homeostasis, which increases the harmful effect on bone metabolism compared to that of physiological hyperprolactinaemia.

Keywords Prolactin receptors · Duodenum · Kidney · Skeletal system · Calcium homeostasis

Introduction

Prolactin (PRL) is a single-chain polypeptide hormone that is synthesized and secreted by lactotrophic cells of the anterior pituitary (AP) gland. PRL is the only pituitary hormone that exhibits an ability for spontaneous hypersecretion. Therefore, it is under constant hypothalamic tonic inhibition.

PRL does not have a target endocrine gland, and it exerts its action via binding to specific prolactin receptors (*Prlr*). The widespread expression of *Prlr* in various tissues may explain the diverse biological functions attributed to PRL in reproduction, metabolism, osmoregulation, immunoregulation

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and behaviour [1]. Besides the well-known *Prlr* expression localisation in mammary glands, the uterus and ovaries, it has also been identified in almost all parts of the gastrointestinal tract (except the rectum) [2], kidneys and the skeletal system [3–5]. These are the target organs for calcium metabolism, and growing evidence in last few decades supports the hypothesis that PRL acts as a calcium-regulating hormone [6, 7].

The oestrogen-dependent effects of PRL, especially on the skeletal system, are widely recognized, but the isolated role of PRL is very often overlooked. However, studies demonstrated that hyperprolactinaemia (caused by AP transplantation) and hypoestrogenaemia (caused by ovariectomy) produced no additive effects [8].

Mothers in states of physiological hyperprolactinaemia (PHP), such as in pregnancy and lactation, require more calcium for foetal growth and milk production. This increased demand for calcium is satisfied via (1) increased intestinal calcium absorption, (2) increased bone calcium mobilization, and/or (3) decreased urinary calcium excretion. Physiologically elevated PRL stimulates intestinal calcium absorption and increases bone turnover [7]. Changes in maternal calcium homeostasis during PHP decrease bone mineral density (BMD) [9]. However, this decreased BMD is transient [10] and completely recovers after weaning [10, 11]. It also produces no long-term detrimental effect on bone health [12, 13]. However, growing evidence indicates that medications used to treat various psychiatric disorders, such as schizophrenia, schizoaffective states, major depressive disorder and bipolar disorder, cause medicamentous hyperprolactinaemia (MHP), which is associated with reduced BMD and increased fracture risk [14–16]. The relationship between MHP and decreased BMD is often attributed to secondary hypogonadism caused by HP [15, 17, 18]. The possible direct role of PRL acting via *Prlr* in target organs involved in calcium homeostasis in causing decreased BMD in MHP is, to our knowledge, not known.

The mechanisms by which hyperprolactinaemia during pregnancy leads to mild and reversible changes in the maternal skeletal system, and why MHP causes more detrimental effects are not completely clear. We performed an experimental study to evaluate differences in *Prlr* gene expression between the duodenum, vertebrae and kidneys during physiological and MHP, which could influence calcium homeostasis.

Materials and methods

Animals

Female Wistar rats (18 weeks old) were obtained from the Laboratory Animal Centre Torlak, Institute for Medical Research, Military Academy of Medicine, Belgrade, Serbia. The experimental study was performed in the Biomedical Research Centre, Medical Faculty, University Nis, Serbia. The study was approved by the Ethics Committee of the Medical Faculty, University Nis, Serbia.

The weight of experimental animals ranged from 290–340 g. Animals were housed under a 12:12-h light-dark cycle (lights on at 06:00) and fed standard chow with normal calcium (1.0% w/w Ca) and water. The room temperature was 23–25 °C, with an average humidity of 50–60%.

Experimental design

Experimental animals were divided into the following groups

P - physiological hyperprolactinaemia (during pregnancy)

Nine pregnant rats (15 weeks old plus 3-week pregnancy, 18 weeks old at the time of sacrifice). The objective for this experimental group was to demonstrate *Prlr* gene expression in the duodenum, vertebrae and kidneys during PHP.

M - medicamentous hyperprolactinaemia, 3-week duration

Ten nulliparous rats (15 weeks old plus 3-week intramuscular administration of sulpiride (10 mg/kg) twice daily, 18 weeks old at the time of sacrifice). The objective for this experimental group was to demonstrate *Prlr* expression in the duodenum, vertebrae and kidneys during sulpiride-induced hyperprolactinaemia.

C - control group

Ten age-matched nulliparous rats (18 weeks old). The objective for this experimental group was to compare the relative expression of *Prlr* genes in the duodenum, vertebrae and kidneys between *P* and *C* and between *M* and *C* to evaluate significant differences in expression during physiological and medicamentous HP.

Laboratory investigations

Blood samples for laboratory measurements were obtained from the left myocardial ventricle through a midline thoraco-abdominal incision followed by euthanasia via exsanguination.

Serum PRL levels were measured in all experimental animals using an enzyme-linked immunosorbent assay (ELISA) kit for PRL. The kit was a sandwich enzyme immunoassay for in vitro quantitative measurements of PRL in rat serum, plasma and other biological fluids (Uscn, Life

Science Inc., Wuhan, P.R. China) with a detection range of 3.12–200 pg/mL. The minimum (min) detectable concentration of rat PRL was 1.03 pg/mL. The standard curve concentrations for this ELISA were 200, 100, 50, 25, 12.5, 6.25, and 3.12 pg/mL.

Vitamin D was measured using a 25-hydroxy vitamin D3 ELISA kit. This assay uses the competitive inhibition enzyme immunoassay technique in rat serum (CUSABIO) with a detection range of 20–100 µg/mL. The min detectable dose of rat vitamin D3 was 5 µg/mL. The intra-assay coefficient of variation was < 10% (precision within an assay CV % < 10%), and inter-assay CV was less than 15% (precision between assays: CV % < 15%).

Parathyroid hormone (PTH) was measured using a rat PTH ELISA kit. This assay uses the quantitative sandwich enzyme immunoassay technique in rat serum, plasma and tissue homogenates (CUSABIO) with a detection range of 6.25–400 pg/mL The min detectable dose of rat PTH was 1.56 pg/mL. The intra-assay coefficient of variation was < 8% (precision within an assay CV % < 8%), and the inter-assay CV was less than 10% (precision between assays: CV % < 10%).

All experimental animals were analysed for serum ionized calcium and inorganic phosphorus. Mineral assays were performed using the following methods: serum ionized calcium was assayed using the potentiometric method, and serum phosphate concentration was measured using the photometric UV test (Beckman Coulter, OLYMPUS analyser).

All rats in each group were housed in single rat metabolic cages to collect 24-h urine samples for calciuresis and phosphouresis, prior to euthanasia. Urine calcium was measured using a photometric colour test, and urine phosphate concentration was measured using a photometric UV test (Beckman Coulter, OLYMPUS analyser).

We measured osteocalcin (OC) and serum procollagen type 1 N-terminal propeptide (P1NP) in all experimental animals. Unfortunately, we could not measure any bone resorption markers in our institution, and we report this deficit as a study limitation.

P1NP was measured using an ELISA kit for P1NP (Uscnk, Life Science Inc.) with a detection range of 31.2–2000 pg/mL. The min detectable concentration of rat P1NP was 10.7 pg/mL. The standard curve concentrations used for this ELISA were 2000, 1000, 500, 250, 125, 62.5, and 31.2 pg/mL.

OC concentrations were measured using an electrochemiluminescence immunoassay N-MID osteocalcin kit (Cobas, Roche) with a detection range of 0.5–300 ng/mL. Values below the detection limit are reported as < 0.5 ng/mL. The intra-assay coefficient of variation was 1.2–4.0%, and the inter-assay CV was less than 1.7–6.5%.

Tissue Preparation

Animals were anaesthetized via intramuscular administration of 0.3 mL 10% ketamine hydrochloride (arylcyclohexylamine derivate) (Ketamidor®, "Richter Pharma", Austria), and a midline thoraco-abdominal incision was performed. The intestinal segment containing the duodenum (10 cm) was removed, rinsed in an ice-cold bath solution and cut longitudinally along the root of the mesentery to expose the mucosa. Duodenal epithelial cells were collected via scraping of the mucosal surface of the duodenal segment using an ice-cold glass slide [19]. Vertebrae L5-6 were excised and cleaned using ice-cold 0.1 M phosphate-buffered saline, pH 7.4. Adhesive connective tissues, muscles, and bone marrow were removed at 4 °C, and all bones were immediately cryopreserved in liquid nitrogen for breaking [4]. Kidney tissue was obtained from each experimental animal, rinsed in an ice-cold bath solution and maintained at −80 °C in RNALater.

RNA isolation and cDNA synthesis

Total RNA was isolated from 20 mg of duodenal mucosal scrapings, vertebrae and kidneys, which were kept at −80 °C in RNALater according to manufacturer's instructions using *RNeasy Mini Kit* (*Qiagen*, Hilden, Germany) and quantified using a *Qubit™Fluorometer* (*Invitrogen*, USA) and *Qubit® RNA Assay Kit* (*Invitrogen*, USA). Aliquots of RNA for qRT-PCR were treated with DNase I (*Applied Biosystems*, *USA*) prior to reverse transcription to eliminate potential DNA contamination. Isolated RNA was stored at −80 °C until further use.

Total RNA (50 ng) of each duodenum and kidney sample was reverse-transcribed using a *High Capacity cDNA Reverse Transcription Kit* (Applied Biosystems, USA), random hexamers (*Applied Biosystems*, SAD) and an RNase inhibitor (*Applied Biosystems*, USA) in a total reaction volume of 20 µL. Reactions were performed in a Mastercycler ep gradient S (Eppendorf, Germany) apparatus. The following thermal profile for reverse transcription was used: 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C. The resultant cDNA was stored at −80 °C until further use.

Total RNA (20 ng) of each vertebra sample was reverse-transcribed using the Sensiscript Kit (Qiagen, Germany), random hexamers (Applied Biosystems, SAD) and an RNase inhibitor (Applied Biosystems, USA) in a total reaction volume of 20 µL. The following thermal profile for reverse transcription was used: 60 min at 37 °C and 5 min at 95 °C. The resultant cDNA was stored in a freezer at −80 °C until further use.

Table 1 Commercial RNA-specific primers used in this experiment

	QuantiTect Primer Assay (Qiagen, Germany)	Detected transcript
β2 microglobulin	Rn_B2m_1_SG, QT00176295	NM_012512
Prolactin receptor gene	Rn_Prlr_vb.1_SG, QT01169518	NM_001034111

Relative quantification of prolactin receptor gene expression using real-time PCR(RT-PCR)

QuantiTect Primer Assays (Qiagen, Germany) were used to amplify *$\beta_2$microglobulin* (endogenous control) and the target gene (Table 1). PCR primers were specific to the long-form prolactin receptor.

Amplification was monitored in a Stratagene Mx3005P system (Agilent Technologies, USA). QRT-PCR was performed using 1 µL of cDNA, QuantiTect Primer Assay and qPCR Master Mix-a (Kapa Biosystems, USA) in a total reaction volume of 10 µL. Negative control, RNA and water instead of cDNA were run in parallel with each set of samples. The following thermal profile was used for the amplification reaction: 3 min of enzyme activation at 95 °C and 40 cycles of amplification (3 s of DNA denaturation at 95 °C, 20 s at 55 °C for primer annealing and 1 s at 72 °C for elongation). The specificity of the amplification products was determined using 2% agarose gel electrophoresis and melting curve analysis. The obtained data were analysed using MxPro™ QPCR software (Agilent Technologies, USA).

Analysis of real-time PCR results

The relative expression levels of mRNA for *Prlr* in experimental groups P and M was determined via comparison to expression of the same mRNA in group C, which was considered the calibrator. The values of relative expression of mRNA for *Prlr* were calculated as $Log_{2.}$

Statistical analysis

Statistical analysis of laboratory results

Data were analysed using SPSS (version 15.0). Continuous (measurable) parameters are presented as mean values (*X*) and the standard deviation and medians with maximum and min values. The Shapiro-Wilk test was used to determine the normality of parameter distribution. Differences were assessed using Student's *t*-test for independent samples if the distribution of parameters was normal, and the Mann-Whitney *U* test was used if parameter distribution was deviated. Student's *t*-test was used for dependent samples (normal distribution), and the Wilcoxon test (deviated distribution) was used to test the statistical significance between continuous parameter values at the beginning and end of the study.

Statistical analysis of real-time PCR results

SPSS 15.0 software package was used for statistical analyses of real-time PCR data. A statistically significant difference within each experimental group was determined using ANOVA analysis. Homogeneity of variance test determined using Levene's method, and Tukey's or Dunnett's tests were used for subsequent post hoc analyses of multiple comparisons. The value of $p \le 0.05$ was considered statistically significant.

Results

PRL concentration in experimental groups

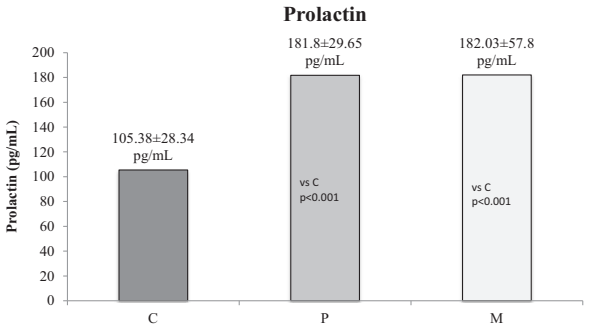
PRL concentrations were significantly higher during the third week of pregnancy (P) compared to C rats (181.80 ± 29.65 pg/mL vs. 105.38 ± 28.34 pg/mL; $p < 0.001$). Significantly increased PRL levels were observed in sulpiride-treated rats (M) compared to age-matched controls, which confirmed the state of MHP (182.03 ± 57.80 pg/mL vs. 105.38 ± 28.34 pg/mL; $p < 0.001$). There was no significant difference in PRL concentration between experimental groups P and M (Fig. 1).

Vitamin D concentration in experimental groups

There was no significant difference in vitamin D levels (reference range 20–100 µg/L) during pregnancy (P) compared to the control group (45.78 ± 1.16 µg/mL vs. 47.12 ± 0.78 µg/mL; $p > 0.05$). No significant changes in vitamin D concentration were identified in the experimental group with MHP (M) comparison with the control group (46.02 ± 1.91 µg/mL vs. 47.12 ± 0.78 µg/mL; $p > 0.05$).

PTH concentration in experimental groups

No statistical significance was observed in PTH concentrations (reference range 6.25–400 pg/mL) in rats with PHP (P) compared with the control group (175.6 ± 32.12 pg/mL vs. 156.11 ± 36.81 pg/mL; $p > 0.05$) or in rats with MHP (M) compared with the control group (168.31 ± 52.18 pg/mL vs. 156.11 ± 36.81 pg/mL; $p > 0.05$).



Experimental groups: P-physiological hyperprolactinaemia; M-medicamentous hyperprolactinaemia; C-control group; P vs. C p<0.001; M vs. C p<0.001.

Fig. 1 PRL concentrations in experimental groups. Experimental groups: P, physiological hyperprolactinaemia; M, medicamentous hyperprolactinaemia; C, control group; P vs. C $p < 0.001$; M vs. C $p < 0.001$

Mineral analysis

Compared to levels in C, serum ionized calcium concentrations were significantly decreased in P (0.5 ± 0.2 mmol/L vs. 1.12 ± 0.04 mmol/L; $p < 0.001$) and increased in M (1.15 ± 0.04 mmol/L vs. 1.12 ± 0.04 mmol/L; $p > 0.05$). A significant difference in calcium concentration was demonstrated between P and M (0.5 ± 0.2 mmol/L vs. 1.15 ± 0.04 mmol/L; $p < 0.001$). Significantly increased phosphorus levels were measured in P compared to C (2.42 ± 0.46 mmol/L vs. 2.05 ± 0.19 mmol/L; $p < 0.05$). Phosphorus concentrations were not significantly altered in M compared with C or P (M vs. C: 2.14 ± 0.48 mmol/L vs. 2.05 ± 0.19 mmol/L; M vs. P: 2.14 ± 0.48 mmol/L vs. 2.42 ± 0.46 mmol/L; $p > 0.05$).

Urinary calcium and phosphorus excretion (measured as daily total excretion) was significantly increased in P compared to the control group (urinary calcium 3.90 ± 0.46 mmol/24 h vs. 3.05 ± 0.58 mmol/24 h; $p < 0.01$; urinary phosphorus 141.15 ± 20.65 mmol/24 h vs. 45.54 ± 7.99 mmol/24 h; $p < 0.001$). Calciuresis was significantly increased in M compared to the control group (4.31 ± 1.11 mmol/24 h vs. 3.05 ± 0.58 mmol/24 h; $p < 0.01$), but phosphouresis was not significantly different (50.58 ± 9.77 mmol/24 h vs. 45.54 ± 7.99 mmol/24 h; $p > 0.05$). (Table 2)

Bone formation markers

Compared to levels in C, OC concentrations were significantly lower in P (9.01 ± 1.09 ng/mL vs. 17.50 ± 2.76 ng/mL; $p < 0.001$) and decreased in M, but without statistical significance (15.28 ± 2.51 ng/mL vs. 17.50 ± 2.76 ng/mL; $p > 0.05$). (Fig. 2)

P1NP concentrations were significantly higher in P (489.22 ± 46.77 pg/mL vs. 361 ± 53.01 pg/mL; $p < 0.001$) and significantly lower in M (309.60 ± 36.74 pg/mL vs. 361.90 ± 53.01 pg/mL; $p < 0.05$) compared to C. The significant decrease of P1NP in M was more obvious compared to P (309.60 ± 36.74 pg/mL vs. 489.22 ± 46.77 pg/mL; $p < 0.001$). (Fig. 3)

Expression of mRNA *Prlr* in the duodenum in experimental groups P and M

Significantly higher *Prlr* mRNA expression was observed in group P compared to group C, and significantly lower *Prlr* expression was observed in group M compared to C ($p < 0.001$). Relative expression of *Prlr* was highest in group P.

Subsequent post hoc analyses revealed that the ratio between the relative expression of *Prlr* mRNA in groups P and C was significantly higher than the ratio between the relative expression of *Prlr* mRNA in groups M and C ($p < 0.001$). (Fig. 4)

Expression of *Prlr* mRNA in vertebrae in experimental groups P and M

Significantly lower *Prlr* mRNA expression was observed in group P compared to C, and significantly higher expression was observed in group M compared to C ($p < 0.001$). Relative expression of *Prlr* was highest in group M.

Subsequent post hoc analyses revealed that the ratio between the relative expression of *Prlr* mRNA in groups P and C was significantly lower than the ratio between the relative expression of *Prlr* mRNA in groups M and C ($p < 0.01$). (Fig. 5)

Expression of *Prlr* mRNA in kidney in experimental groups P and M

Significantly lower expression of *Prlr* mRNA was observed in group P compared to C, and significantly higher expression was observed in group M compared to C ($p < 0.01$). The relative expression of *Prlr* was highest in group M.

Subsequent post hoc analyses revealed that the ratio between the relative expression of *Prlr* mRNA in groups P and C was significantly lower than the ratio between the relative expression of *Prlr* mRNA in groups M and C ($p < 0.01$). (Fig. 6)

Discussion

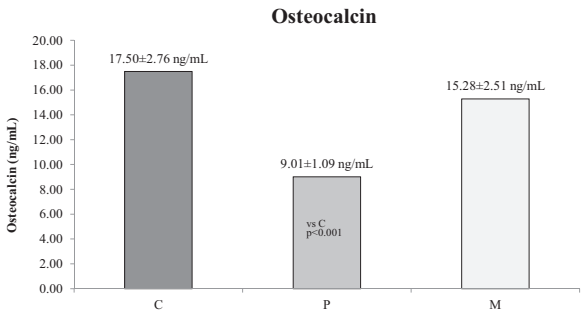
Increased PRL concentration in experimental groups

Maternal PRL levels begin rising at 6–8 weeks of gestation and progressively increase to 200–300 ng/mL at term [19]. Indirect evidence suggests that high oestrogen

Table 2 Mineral analysis in the experimental groups

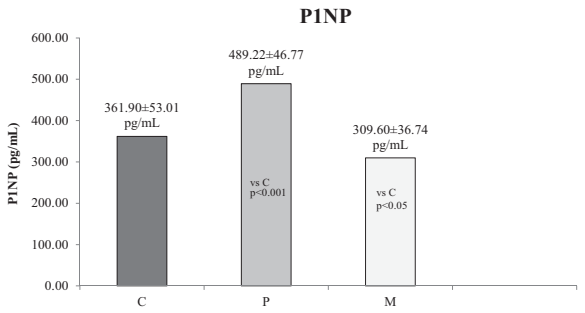
Experimental groups	P-group (physiological hyperprolactinemia)	M-group (medicamentous hyperprolactinemia)	C-group (control group)
Mineral analysis			
s-Ca + + (mmol/L)			
X±SD	0.5 ± 0.2 [a]	1.15±0.04 [b]	1.12 ± 0.04
s-P (mmol/L)			
X±SD	2.42 ± 0.46 [c]	2.14 ± 0.48	2.05 ± 0.19
u-Ca (mmol/24 h)			
X±SD	3.90 ± 0.46 [a]	4.31 ± 1.11 [d]	3.05 ± 0.58
u-P (mmol/24 h)			
X±SD	141.15 ± 20.65 [a]	50.58 ± 9.77	45.54 ± 7.99

Statistical significance: [a] P vs. C $p < 0.001$; [b] M vs. P $p < 0.001$; [c] P vs. C $p < 0.05$; [d] M vs. C $p < 0.01$.



Experimental groups: P-physiological hyperprolactinaemia; M-medicamentous hyperprolactinaemia; C-control group; P vs. C p<0.001.

Fig. 2 OC concentration in experimental groups. Experimental groups: P, physiological hyperprolactinaemia; M, medicamentous hyperprolactinaemia; C, control group; P vs. C $p < 0.001$

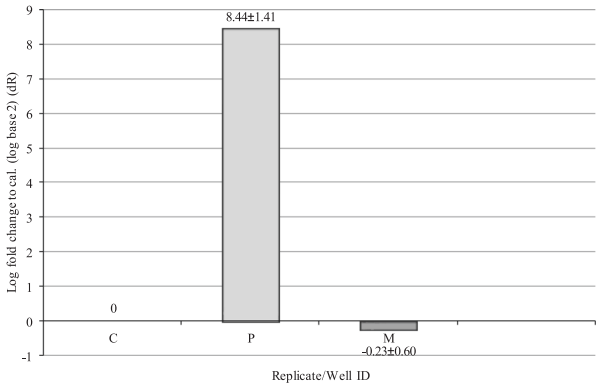


Experimental groups: P-physiological hyperprolactinaemia; M-medicamentous hyperprolactinaemia; C-control group; P vs. C p<0,001; M vs. C p<0.05.

Fig. 3 P1NP concentration in experimental groups. Experimental groups: P, physiological hyperprolactinaemia; M, medicamentous hyperprolactinaemia; C, control group; P vs. C $p < 0.001$; M vs. C $p < 0.05$

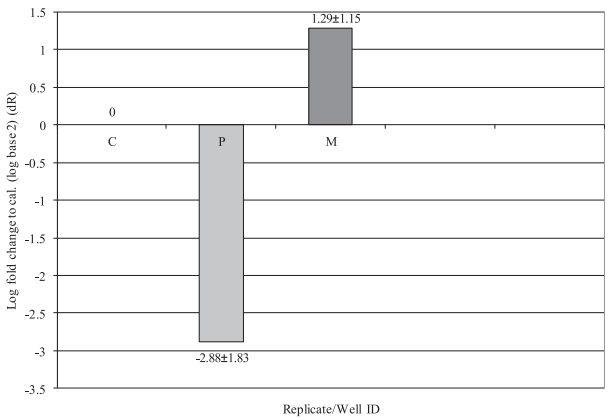
concentrations stimulate lactotroph size and proliferation, which leads to pituitary enlargement and increased PRL release [20, 21].

Various normal concentrations of rat serum PRL are obtained depending on the methodology. Radioimmunoassay kits determine PRL levels in the range of 0.1–20 ng/mL or 0.8–50 ng/mL, and ELISA kits exhibit



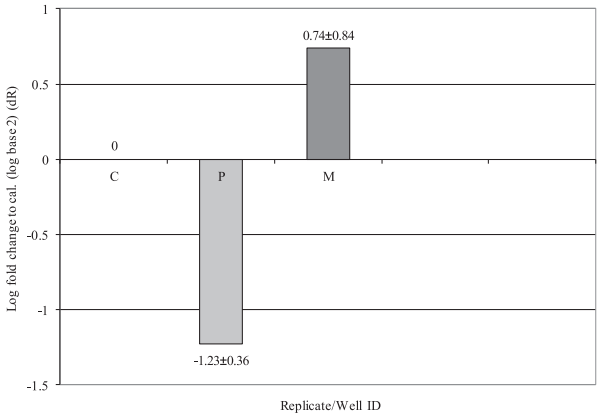
Experimental groups: P-physiological hyperprolactinaemia; M-medicamentous hyperprolactinaemia; C-control group; $\log_2$(P/C) vs $\log_2$(M/C) p<0.001

Fig. 4 Relative expression of mRNA *Prlr* in the duodenum during physiological and medicamentous hyperprolactinaemia. Experimental groups: P, physiological hyperprolactinaemia; M, medicamentous hyperprolactinaemia; C, control group; $\log_2$(P/C) vs. $\log_2$(M/C) $p < 0.001$



Experimental groups: P-physiological hyperprolactinaemia; M-medicamentous hyperprolactinaemia; C-control group; $\log_2$(P/C) vs. $\log_2$(M/C) p<0.01.

Fig. 5 Relative expression of *Prlr* mRNA in vertebrae during physiological and medicamentous hyperprolactinaemia. Experimental groups: P, physiological hyperprolactinaemia; M, medicamentous hyperprolactinaemia; C, control group; $\log_2$(P/C) vs. $\log_2$(M/C) $p < 0.01$



Experimental groups: P-physiological hyperprolactinaemia; M-medicamentous hyperprolactinaemia; C-control group; $\log_2$(P/C) vs. $\log_2$(M/C) p<0.01.

Fig. 6 Relative expression of *Prlr* mRNA in kidney during physiological and medicamentous hyperprolactinaemia. Experimental groups: P, physiological hyperprolactinaemia; M, medicamentous hyperprolactinaemia; C, control group; $\log_2$(P/C) vs. $\log_2$(M/C) $p < 0.01$

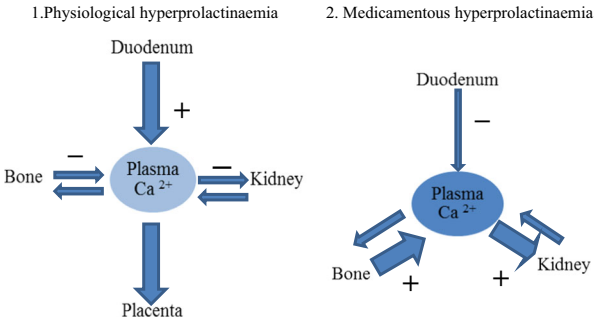


Fig. 7 Hypothetical model of the role of PRL in calcium homeostasis during physiological and medicamentous hyperprolactinaemia

higher variability in referent ranges (0.125–50 ng/mL, 0.41–100 ng/mL and 3.12–200 pg/mL). Hyperprolactinaemia is always expected in pregnant rats regardless of the methodology used. The average length of pregnancy is 19–22 days in Wistar rats. PRL levels are elevated during the first 10–12 days because of daily nocturnal and diurnal PRL surges [20]. Placental lactogens increase during the second half of rodent pregnancy, which leads to maternal PRL suppression [22]. An increased oestrogen/progesterone ratio triggers a large PRL surge just prior to parturition [23]. This antepartum PRL surge plays a dual role. It participates in the final maturation of the mammary gland in preparation for lactation, and it affects the onset of maternal behaviour [24]. Our study results confirmed the expected significant increase in PRL levels at the end of the third week of pregnancy. These PRL levels are considered PHP.

Sulpiride is a second generation antipsychotic. These medications are generated to achieve stronger dopamine blockade, which results with neuroleptic syndrome, and suppress extrapyramidal syndrome. The use of sulpiride as an antipsychotic is often followed by increased PRL levels, which are maintained during the entire time of therapy [25]. Sulpiride is also used as an antidopaminergic gastrointestinal prokinetic for the management of motor disorders of the upper gastrointestinal tract, including functional dyspepsia, gastric stasis of various origins and emesis [26]. The prokinetic effect is mediated via blockade of enteric D_2 receptors, and the anti-emetic effect is the result of central D_2 receptor antagonism. The pituitary gland is outside the blood-brain barrier, and hyperprolactinaemia occurs as an adverse effect of all antidopaminergic prokinetics, including sulpiride. Significantly increased PRL levels in sulpiride-treated rats were confirmed in our study, and was considered as MHP.

There are no data on the hyperprolactinaemia influence on the renal clearance of PRL per se, including different *Prlr* expression in the kidney during medicamentous and PHP. However, decreased renal clearance of PRL is observed during impaired renal function, which leads to increased PRL levels [27]. Another underlying mechanism causing hyperprolactinaemia in chronic renal failure is lactotroph resistance to dopamine suppression and increased PRL secretion [28]. Both pathways support impaired renal function as one of the pathophysiological causes of hyperprolactinaemia. The animals used in the present experiment were healthy subjects without chronic kidney disease. Therefore, PRL clearance should not be diminished.

Changes in mineral analysis during PHP and MHP

Serum ionized calcium

The significant decrease in ionized calcium in our study during PHP is consistent with several previous studies in animal models, which reported a decrease in ionized calcium during late pregnancy [29, 30]. Rapid foetus growth in late pregnancy may lead to an excess of the maternal capacity to maintain a normal serum calcium level and result in decreased ionized calcium. There were no significant changes in ionized calcium during MHP, which is consistent with limited literature data [31, 32].

Experimental animal models using radioactive Ca (calcium gluconate containing 2 mCi (1 Ci = 37 GBq) 45Ca) demonstrated that endogenous PRL increased food consumption, calcium absorption, and bone calcium turnover during states of PHP, like pregnancy and lactation, which apparently increased calcium availability for foetal development and milk calcium secretion [33].

Phosphorous

Serum phosphate levels are generally reported as normal throughout pregnancy in humans and animals [34, 35]. However, phosphate levels increased significantly during

PHP, which may be a compensatory response to decrease calcium and may be explained by the fact that dietary phosphorus is absorbed almost twice as efficiently as dietary calcium [36].

Urinary calcium and phosphorus excretion

The increases in urinary calcium and phosphorus excretion during pregnancy were similar to previous reports and are likely a consequence of increased calcium absorption and elevation of glomerular filtration rate (GFR) during pregnancy, which exceed the re-absorptive capacity of the kidney [34, 35, 37].

Previously, reported data demonstrated that a high-calcium diet (2.0% w/w Ca) modulated the effects of PRL on bone formation, resorption, or calcium deposition [4]. Tudpor et al. [38] demonstrated that a high-calcium diet (2.0% w/w Ca) reduced the stimulatory action of PRL on duodenal calcium absorption. All experimental animals in the present study were fed standard chow with normal calcium (1.0% w/w Ca) to avoid the potential influence of high Ca intake on urinary calcium excretion. The experimental group of pregnant rats received the same diet throughout the experiment. A significant increase in urinary calcium excretion was verified in animals with both physiological and drug-induced hyperprolactinaemia compared to control animals. A previous study demonstrated that chronic hyperprolactinaemia caused by implanted AP glands under the kidney capsule also increased urinary calcium excretion [39].

Notably, urinary calcium excretion was significantly higher in the MHP compared to the experimental group with PHP despite similar PRL levels and identical calcium intake. Possible explanations include differential *Prlr* expression in the kidney, variance in postreceptor pathways or synergistic actions of dopamine and PRL in the kidney, which resulted in the higher calciuresis during MHP. Future studies will reveal the precise mechanism of this discrepancy.

Changes in bone turnover markers during PHP and MHP

Features, such as the fast response to changes in skeletal homeostasis and its origin from osteoblasts, make OC a reliable marker of bone turnover. An increase in OC correlates with increased bone formation. The decrease in OC during pregnancy in our study is consistent with previous findings. The decrease in OC may be related to haemodilution, foetal contribution [37] increased renal degradation [40] or the absence of normal levels during pregnancy [41]. The data on OC during MHP are controversial. Increased OC was reported in women receiving therapy for depressive disorders [31, 42], and decreased OC was reported in patients with chronic schizophrenia receiving antipsychotic treatment [43].

P1NP is a very valuable and precise marker of osteosynthesis. Our study results are consistent with the limited, previously reported data of increased P1NP concentrations in late pregnancy [44].

The discrepancies between OC and P1NP serum concentrations, verified in our study, could be explained by the fact that these two biomarkers of osteosynthesis reflect different aspects of osteoblastic activity. Most OC production occurs during the mineralization phase, and P1NP reflects osteoblast proliferation [42]. Higher foetal demands for calcium may be one reason for the temporary cessation of mineralization in the maternal skeletal system, which results in lower OC concentration. On the other hand, increased P1NP in late pregnancy is encouraging data because it reveals increased osteoblast proliferation, which will certainly aid in the faster recovery of bone loss after pregnancy.

Analysis of *Prlr* expression in the duodenum during PHP and MHP

Intestinal calcium absorption occurs via passive and active pathways. The major route of calcium absorption is through passive paracellular channels, which is mainly determined by calcium concentration gradients across the intestinal epithelia. Recent studies demonstrated that PRL increases paracellular calcium permeability and passive calcium transport in the intestine [45, 46]. On the other hand, active intestinal calcium transport is a transcellular process that involves three steps: (1) apical entry of calcium through apical calcium channels; (2) calcium transfer through the enterocyte cytoplasm; and (3) basolateral extrusion of calcium via the calcium pump, Ca^{2+}-ATPase [47]. The agent 1, 25-dihydroxyvitamin D_3 is considered to be the most important regulating hormone of the active pathway of calcium absorption, and PRL stimulates active intestinal calcium absorption [48] in vitamin D-deficient rats [5, 49]. Studies performed using the chamber technique confirmed a direct PRL effect on duodenal transcellular calcium transport [50]. Pregnancy and lactation increase the demand for calcium, and active intestinal calcium absorption becomes more important [7, 19]. Our study results confirmed a significant increase in *Prlr* expression in duodenum during PHP compared to an age-matched control group. This upregulation of *Prlr* in duodenum in late pregnancy allows a more intense direct PRL enhancement of Na(+)K(+)- and Ca(2+)-ATPase activities in duodenal epithelial cells [51] and increase in the active pathway of calcium absorption. Similar PRL concentrations were documented in pregnant and sulpiride-treated rats, and the duration of PHP and MHP was identical (3 weeks). The present study revealed a significant decrease in *Prlr* expression in duodenum in rats with MHP compared to an age-matched control group. To our knowledge, no experimental studies investigated *Prlr*

expression in the duodenum during MHP. Our study results demonstrate, for the first time, that the ratio between relative mRNA expression of *Prlr* in the PHP and control groups was significantly higher than the ratio between the relative expression of *Prlr* mRNA in rats with MHP and age-matched controls. Previous data demonstrated an important role for PRL in passive and active intestinal calcium absorption. Therefore, the upregulation of *Prlr* in duodenum during pregnancy in our study may be a physiological response that helps the mother preserve calcium homeostasis during a period with increased calcium demands. However, the downregulation of *Prlr* in the duodenum during MHP may diminish the role of PRL in intestinal calcium absorption.

Analysis of *Prlr* expression in vertebrae during PHP and MHP

The idea of direct role for PRL in the skeletal system arose from Bataille-Simoneau et al., [3] who discovered *Prlr* in human osteosarcoma cells. Further investigation verified the presence of *Prlr* in osteoblasts and their absence in osteoclasts [5]. RT-PCR analysis revealed *Prlr* expression in different bones, such as tibiae, femurs and vertebrae [4]. A direct influence of PRL on osteoblasts, such as slower differentiation and reduced total osteoblast number, was verified in vitro [52]. Lower expression of alkaline phosphatase and OC mRNA in osteoblasts exposed to direct PRL influence decreased mineralization in vitro [52–54]. Our study results confirmed expression of the long-form *Prlr* in the vertebrae of each experimental group. Significantly lower *Prlr* expression was demonstrated in the vertebrae during pregnancy. Significantly decreased *Prlr* expression in vertebrae reduces the direct PRL influence and protects the mother's skeletal system from further calcium loss and bone demineralization. These results may explain the minor and reversible BMD loss during pregnancy [9, 55, 56].

Significantly increased *Prlr* expression in vertebrae was detected in the experimental group with MHP compared to controls. Our study results demonstrate, for the first time, that the ratio between the relative expression of *Prlr* mRNA in the MHP and control groups was significantly higher than the ratio between the relative expression of *Prlr* mRNA in the rats with physiological HP and age-matched controls. This *Prlr* upregulation in vertebrae during MHP allows a more intense effect of PRL, which leads to slower differentiation and smaller numbers of osteoblasts [52]. The downregulation of *Prlr* in the duodenum during MHP will close the "duodenal doors" for calcium absorption. The upregulation of *Prlr* in the skeletal system may be a compensatory attempt, i.e. an open window to provide calcium and maintain calcium homeostasis, which increases the detrimental effects on bone metabolism compared to PHP.

Analysis of *Prlr* expression in kidney during PHP and MHP

Different methods, such as immunohistochemical staining, radioligand receptor assays and *real-time PCR*, verified *Prlr* expression in the kidneys of different animals [57, 58] and humans [59].

The increased urinary calcium and phosphorus excretion during pregnancy may be a consequence of increased calcium absorption and elevation in GFR during pregnancy, which exceeds the re-absorptive capacity of the kidney [34, 35, 37]. However, the same mechanism cannot explain the higher hypercalciuria in sulpiride-induced hyperprolactinaemia and suggests an indirect PRL influence on calciuresis because of the role of PRL in urinary sodium excretion. The main physiological role of Na+/K + ATPase is sodium reabsorption. This enzyme pushes potassium out of the cell and returns sodium into nephron epithelial cells. Even though there have been some discrepancies in past results, whether PRL increases natriuresis or induces an antidiuretic or antinatriuretic response is not clear, but recent studies clearly demonstrate that PRL inhibits the Na+/K activity of + ATPase in the proximal section of renal tubules [60]. PRL acts as a natriuretic hormone in the inhibition of Na (+)/K(+)-ATPase activity [61]. Increased natriuresis indirectly leads to increased calcium elimination. Our study results demonstrated a significant decrease in *Prlr* expression in kidneys during pregnancy compared to MHP. The downregulation of *Prlr* should increase Na(+)/K (+)-ATPase activity and decrease natriuresis. This decreased sodium excretion would indirectly aid calcium tubular reabsorption. On the other hand, upregulation of *Prlr* in the experimental group with MHP likely resulted in the opposite reaction and decreased Na(+)/K(+)-ATPase activity, which increased natriuresis and indirectly increased calciuresis.

The role of PRL in the regulation of sodium and water transport is one of its least understood roles. The influence of drug-induced hyperprolactinaemia and PHP on tubular calcium reabsorption is less well-known. Further investigations are necessary to reveal the precise role of PRL in urinary calcium excretion. Based on results of *Prlr* expression in the duodenum, vertebrae and kidney, we made a hypothetical model for PRL role in calcium homeostasis during physiological and medicamentous hyperprolactinemia shown in Fig. 7.

Conclusion

Increased mRNA expression of *Prlr* in the duodenum during pregnancy leads to higher intestinal calcium absorption. Decreased *Prlr* expression in the kidney may be

a compensatory attempt to prevent calcium loss via the kidney. The decreased *Prlr* expression in vertebrae reduces the direct effect of PRL and protects the maternal skeletal system from demineralisation. The decreased duodenal expression of *Prlr* during MHP may explain the diminished intestinal calcium absorption. The increased expression of *Prlr* in the kidney may be one of the factors responsible for the increased calciuresis. If "intestinal doors" for calcium absorption are closed, then the skeletal system becomes an "open window" for the increased direct effect of PRL. Increased *Prlr* gene expression in the vertebrae allows PRL to remove calcium from the skeletal system to maintain calcium homeostasis and increases the harmful effect on bone metabolism compared to the effects of PHP.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The Ethical Committee of the Medical Faculty, University Nis, Serbia approved the study.

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