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EXPERIMENTAL STUDY

Puerarin affects bone biomarkers in the serum of rats with intrauterine growth restriction

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Abstract

OBJECTIVE: To investigate serum bone biomarkers in rats with intrauterine growth restriction (IUGR) in order to determine the effects of puerarin on bone metabolism.

METHODS: A rat model of IUGR was induced using a low protein diet during pregnancy. The offspring were given puerarin or an identical volume of saline via subcutaneous abdominal injection. All rats were studied at 1, 3, and 8 weeks of age. Serum biomarkers of bone formation, including insulin-like growth factor-1 (IGF-1), bone-specific alkaline phosphatase (BALP), osteocalcin (OC), osteoprotegerin (OPG), receptor-activator of nuclear factor-κB ligand (RANKL), as well as blood levels of calcium and phosphorus were measured.

RESULTS: Serum BALP, OPG, IGF-1, and OC levels, as well as the OPG/RANKL ratio, were lower in the IUGR group compared with the control group at 1 week of age (P = 0.024, 0.011, 0.014, 0.004, and 0.002, respectively). At 3 weeks of age, the serum BALP and OC levels were higher in the protein-restricted group compared with the control group (P = 0.003 and 0.001, respectively). A comparison between the IUGR plus puerarin intervention group and the IUGR group revealed differences in the levels of BALP and IGF-1 at 3 weeks of age (P = 0.008 and 0.003, respectively). In addition, serum OPG and OC levels and the OPG/RANKL ratio were higher at 8 weeks of age (P = 0.044, 0.007, and 0.016, respectively). No differences in serum calcium and phosphorus levels were observed among the three groups.

CONCLUSION: Our study demonstrates that the bone microenvironment of the fetus can be altered by a low protein maternal diet and that puerarin can reverse these effects. These results indicate that the nutritional environment plays an important role in early skeletal development and that the bone turnover of IUGR rats can be altered by puerarin treatment.

Key words: Puerarin; Fetal growth retardation; Osteogenesis; Bone biomarker

INTRODUCTION

In humans, the development of the fetus can have major influences on the adult phenotype. A good example of this phenomenon is seen in intrauterine growth restriction (IUGR), which can be defined as when the estimated fetal weight (EFW) is below the 10th percentile of healthy fetus of the same gestational age. Causes of IUGR include malnutrition, placental insufficiency, and congenital anomalies. IUGR affects approximately 10%-15% of pregnant women.[1] Fetuses with IUGR

have a higher risk of perinatal morbidity, mortality and long-term sequelae, including osteoporosis, obesity, insulin resistance, hypertension, and brain injury.

Bone formation requires adequate supplies of energy, amino acids, minerals, and vitamins. Some studies have indicated that the intrauterine environment plays an important role in skeletal development.[2] In recent years, many studies have reported that protein deficiencies during pregnancy have an adverse effect on bone growth. Mehta *et al* [3] reported that maternal protein restriction during pregnancy results in reduced bone area and bone mineral content (BMC). Lanham *et al* [4] also demonstrated that a low protein maternal diet affects the bone structure of female offspring when they reach an elderly age using micro-computed tomography (micro-CT) and mechanical testing. Thus, bone growth may have been affected by IUGR, which was induced by protein deficiency during pregnancy.

In recent years, estrogen replacement therapy has been considered an effective approach for preventing age-related bone loss in females.[5] Puerarin [4H-1-benzopyran-4-one,8-b-D-glucopyranosyl-7-hydroxy-3-(4-hydroxy-phenyl)] is a major phytoestrogen, isolated from Gegen (*Radix Puerariae Lobatae*), which binds to estrogen receptors and has weak estrogenic activity. Wang *et al*.[6] demonstrated that puerarin reduces the loss of bone density that occurs in ovariectomized mice. Wong *et al* [7] also demonstrated that puerarin induces local increases in new bone formation in a collagen matrix.

This study was based on the hypothesis that serum bone biomarkers may differ between rats with IUGR and healthy controls. We further hypothesized that puerarin treatment could promote recovery of serum bone biomarkers to normal levels. Serum levels of insulin-like growth factor-1 (IGF-1), osteocalcin (OC), bone specific alkaline phosphatase (BALP), osteoprotegerin (OPG), and receptor-activator of nuclear factor kappa beta ligand (RANKL) were measured to determine the association between these biomarkers and IUGR. In addition, circulating levels of calcium and phosphorus were evaluated.

MATERIALS AND METHODS

Animals and treatments

All experimental designs and procedures received approval by the Animal Ethics Committee of Central South University (Changsha, China). Specific pathogen free -grade Sprague-Dawley rats [18 females, 9 males; 3 months of age, weighing (220 ± 20) g], purchased from the Experimental Animal Center of Central South University [certificate of quality No. SCXK (xiang) 2009-0012], were housed individually at 25 ℃, with a 12∶12 h light-dark cycle. Successful mating was confirmed after observing sperm in a vaginal smear; the day of successful mating day was used as day zero of gestation. After gestational day 0, pregnant dams were housed individually and were randomly allocated to either a normal protein diet containing 21% protein (w/w), for the control group, or a low protein diet containing 10% protein (w/w) for both the IUGR and the IUGR plus puerarin intervention groups; both diets had the same caloric value.[4,8] Offspring of both the IUGR and IUGR plus puerarin intervention groups were randomly divided into two subgroups using a random number table. One group received 50 $mg \cdot kg^{-1} \cdot d^{-1}$ of puerarin (Tianjin Pharmaceutical Group Co., Xinzheng Ltd., Xinzheng, Henan, China) via subcutaneous abdominal injection for 1 week, whereas the other group received an injection of the same volume of saline. Experimental samples were harvested after 1, 3, and 8 weeks. Blood was collected in pyrogen-free tubes after centrifugation. The supernatants from the serum samples were frozen at − 80 °C until the time of the assay.

Plasma OPG levels were measured using an enzyme-linked immunosorbent assay (ELISA; SEA108Ra, Cloud-clone Co., Houston, TX, USA) with a detection range of 0.156-10 ng/mL and a sensitivity of 0.053 ng/mL. Plasma IGF-1 levels were also measured using an ELISA (SEA050Ra, Cloud-clone Co., Houston, TX, USA) with a detection range of 78.125-5000 pg/mL and a sensitivity of 30 pg/ml. Plasma osteocalcin levels were measured using an ELISA (SEA 471Ra, Cloud-clone Co., Houston, TX, USA) with a detection range of 15.625-1000 pg/mL and a sensitivity of 6.3 ng/mL. Plasma BALP levels were measured using an ELISA (SEB 091Ra, Cloud-clone Co., Houston, TX, USA) with a detection range of 0.781-50 ng/mL and a sensitivity of 0.28 ng/ml. Plasma RANKL levels were measured using an ELISA (SEA855Ra, Cloud-clone Co., Houston, TX, USA) with a detection range of 15.625-1000 ng/mL and a sensitivity of 5.3 ng/mL. Serum calcium and phosphorus levels were measured using the ARCHITECT c8000 system (Abbott Co., Chicago, IL, USA).

Statistical analysis

The data are represented as the mean ± standard deviation ($\bar{x} \pm s$). Multiple samples were compared using analysis of variance. Several sets of multiple samples were analyzed *via* several pairwise comparisons using the analysis of variance least significant difference method. Sample rates were compared using the χ^2 test. All computations were performed using SPSS 17.0 software (SPSS Inc., St. Louis, MO, USA). Differences with $P < 0.05$ were considered statistically significant.

RESULTS

The birth weights of offspring whose mothers were fed a restricted protein diet (i.e., the IUGR and IUGR plus puerarin intervention groups) met the criteria for IUGR and were significantly lower than those of the control group ($P > 0.001$). After 1 week, the body

weights of rats in both the IUGR and the IUGR plus puerarin intervention groups were also lower than that of the control group (P = 0.001 and P = 0.002, respectively). After 3 weeks, the body weights of rats in the IUGR plus puerarin intervention group increased, although they were not significantly different from that of the IUGR group (P = 0.081). After 8 weeks, the body weights of rats in the IUGR plus puerarin intervention group were significantly higher than that of the IUGR group (P = 0.032) (Figure 1).

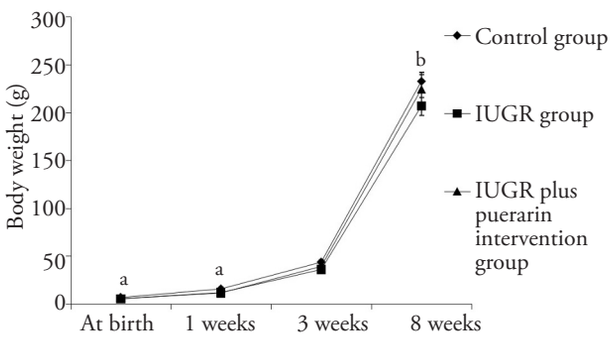

Figure 1 Line chart of the body weights of the three groups at birth, and at 1, 3 and 8 weeks of age

Control group was treated with only physiological saline; whereas both the IUGR and IUGR plus puerarin groups, who were offspring of mothers administered low protein diets during pregnancy, were treated with physiological saline or puerarin, respectively. IUGR: intrauterine growth restriction. $^aP < 0.05$, control group vs IUGR group; $^bP < 0.05$, IUGR group *vs* IUGR plus puerarin intervention groups.

ferences in serum BALP, OPG, OPG/RANKL, IGF-1, or OC levels were observed between the two groups at 8 weeks of age ($P > 0.05$).

At 3 weeks of age, serum BALP and IGF-1 levels were significantly higher in IUGR rats administered puerarin compared with IUGR rats that did not receive intervention (P = 0.008 and 0.003, respectively). At 8 weeks of age, serum OPG and OC levels, as well as the OPG/RANKL ratios of rats administered puerarin were higher than those of IUGR group rats, while the levels of BALP and IGF-1 continued to increase in IUGR groups.

At 1 week of age, the IGF-1 levels of both the IUGR and the IUGR plus puerarin intervention groups were significantly lower than those of the control group (P = 0.001 and 0.002, respectively). At 3 weeks of age, the IGF-1 levels of the IUGR plus puerarin intervention group significantly increased more than those of both the control and IUGR groups (P = 0.003 and 0.010, respectively). However, at 8 weeks of age, there were no differences observed among the three groups (P = 0.656).

At 1 week of age, the OC levels of the control group were also significantly higher than those of both the IUGR and the IUGR plus puerarin intervention groups (P = 0.004 and 0.037, respectively). However, at 3 weeks of age, the OC levels of the control group were significantly lower than those of both the IUGR and IUGR plus puerarin intervention groups (P = 0.001 and 0.003, respectively). The OC levels of the IUGR plus puerarin intervention group continued to increase and were the highest among the three groups at 8 weeks of age. The differences observed between comparisons of the IUGR plus puerarin intervention group with the control group and the IUGR group were statistically significant (P = 0.013 and 0.007, respectively).

The BALP levels of the control group were higher than

Table 1 Serum levels of bone markers from 1-, 3- and 8-week-old mice from all the three groups (n = 6, $\bar{x} \pm s$)

Time point	Group	n	IGF-1 (μg/L)	Osteocalcin (ng/L)	BALP (ng/L)	OPG (ng/L)
1 week	Control	6	7.91±0.32	413.98±6.77	3.21±0.35	549.77±26.42
	IUGR	6	6.36±0.19[a]	343.77±23.85[a]	2.58±0.19[a]	470.32±8.42[a]
	IUGR plus puerarin intervention	6	6.73±0.89	371.87±22.42	2.61±0.15[b]	439.80±58.22
3 weeks	Control	6	7.20±0.53	325.45±16.65	3.25±0.28	548.78±39.55
	IUGR	6	7.59±0.22	396.84±13.20[a]	4.12±0.22[a]	464.57±44.64
	IUGR plus puerarin intervention	6	8.95±0.52[b]	386.34±17.08	3.95±0.44	505.98±58.21
8 weeks	Control	6	7.29±0.29	372.20±39.67	3.59±0.15	515.10±45.83
	IUGR	6	7.69±0.67	360.27±1.92	3.98±0.04[a]	472.85±47.04
	IUGR plus puerarin intervention	6	7.10±1.03	443.61±17.75[b]	3.63±0.15[b]	553.18±14.02[b]

Notes: control group was treated with only physiological saline; whereas both the IUGR and IUGR plus puerarin groups, who were offspring of mothers administered low protein diets during pregnancy, were treated with physiological saline or puerarin, respectively. IUGR: intrauterine growth restriction; IGF-1: insulin-like growth factor-1; BALP: bone-specific alkaline phosphatase; OPG: osteoprotegerin. $^aP < 0.05$, *vs* the control group; $^bP < 0.05$, *vs* the IUGR group.

The values for each bone marker examined are presented in Tables 1 and 2. A comparison between both the IUGR and control groups revealed that serum levels of the bone biomarkers BALP, OPG, OPG/RANKL, IGF-1, and OC were significantly lower than those of the control group at 1 week of age (P = 0.024, 0.011, 0.002, 0.014, and 0.004, respectively). After 3 weeks, serum BALP and OC levels in the IUGR group were significantly higher than those of the control group (P = 0.003 and 0.001, respectively). No significant dif-

Table 2 Serum levels of bone markers and OPG/RANKL ratios from 1-, 3-, and 8-week-old mice from all three groups ($\bar{x} \pm s$)

Time point	Group	*n*	RANKL (pmol/L)	OPG/RANKL	Blood calcium (mmol/L)	Blood phosphorus (mmol/L)
1 week	Control	6	175.09±10.63	3.14±0.08	2.40±0.27	4.19±0.64
	IUGR	6	201.34±7.24[a]	2.34±0.11[a]	2.48±0.18	4.44±1.20
	IUGR plus puerarin intervention	6	202.84±11.87	2.17±0.32	2.36±0.20	4.31±1.04
3 weeks	Control	6	185.17±10.10	2.97±0.34	2.60±0.14	3.10±0.41
	IUGR	6	191.24±3.02	2.43±0.26	2.50±0.06	3.20±0.12
	IUGR plus puerarin intervention	6	165.31±7.95[b]	3.08±0.49	2.57±0.03	2.94±0.16
8 weeks	Control	6	176.02±9.20	2.93±0.26	2.47±0.16	2.80±0.49
	IUGR	6	191.24±3.24[a]	2.48±0.28	2.49±0.09	2.87±0.28
	IUGR plus puerarin intervention	6	170.67±8.47[b]	3.25±0.16[b]	2.57±0.05	2.62±0.33

Notes: control group was treated with only physiological saline; whereas both the IUGR and IUGR plus puerarin groups, who were offspring of mothers administered low protein diets during pregnancy, were treated with physiological saline or puerarin, respectively. IUGR: Intrauterine growth restriction; OPG: osteoprotegerin; RANKL: receptor-activator of nuclear factor-κB ligand. [a]$P < 0.05$, control group *vs* IUGR group. [b]$P < 0.05$, IUGR group *vs* IUGR plus puerarin intervention group.

those of the IUGR and the IUGR plus puerarin intervention groups ($P = 0.020$ and 0.024, respectively) at 1 week of age; however, they were significantly elevated in both the IUGR and the IUGR plus puerarin intervention groups at 3 weeks of age, but decreased again at 8 weeks.

The OPG levels of all groups exhibited a similar pattern to that observed for IGF-1. At 1 week of age, the OPG levels of both the IUGR and the IUGR plus puerarin intervention groups were significantly lower than those of the control group ($P = 0.040$ and 0.011, respectively). At 3 weeks of age, the OPG levels of the IUGR plus puerarin intervention group were elevated; the OPG levels of the IUGR plus puerarin intervention group were significantly higher than those of the IUGR group at 8 weeks of ages ($P = 0.044$).

The RANKL levels of the groups exhibited a pattern that was opposite to that observed for OPG levels, with the levels of the control group being lower than those of both the IUGR and the IUGR plus puerarin intervention groups ($P = 0.019$ and 0.015, respectively). At 3 weeks of age, the RANKL levels of the IUGR plus puerarin intervention group decreased significantly. The RANKL levels of the IUGR plus puerarin intervention group were lower than those of the IUGR group at 3 and 8 weeks of age ($P = 0.006$ and 0.016, respectively).

At 1 week of age, the OPG / RANKL ratios of the IUGR group and the IUGR plus puerarin intervention group were lower than that of the control group ($P = 0.001$ and 0.002, respectively). At 3 weeks of age, the OPG/RANKL ratio of the IUGR plus puerarin intervention group was significantly higher than those of both the control and IUGR groups ($P = 0.003$ and 0.010, respectively). However, at 8 weeks of age, there were no significant differences observed among the three groups ($P > 0.05$).

Comparisons among the three groups revealed no significant differences in serum calcium and phosphorus levels ($P > 0.05$).

DISCUSSION

This study demonstrated that the bone microenvironment of offspring can be altered by a low protein maternal diet during pregnancy and that puerarin can reverse these effects. Maternal nutrition during pregnancy not only affects fetal growth but also has long-term effects on the growth and development of the child, including their bones. Low birth weight is one of the most direct manifestations of IUGR. Birth weight has been demonstrated to be a predictor of adult bone mass and skeletal size.[9] Thus, IUGR induced by poor maternal nutrition during pregnancy could affect adult bone mass and skeletal size.

Maternal malnutrition during pregnancy affects bone growth

The growth hormone (GH) / IGF-1 axis is a key endocrine pathway. In the blood, insulin-like growth factors (IGFs) bind to IGF-binding proteins (IGFBPs),which determines the bioavailability of these factors and regulates the interaction between IGFs and insulin-like growth factor-1 receptor (IGF-1R).[10] Studies in animal models and in humans established critical roles for IGFs in skeletal growth and development. Bikle *et al* [11] reported that $Igf1^{-/-}$ mice exhibit a 24% reduction in cortical bone size and a shortened femoral length. Human patients with IGF1 gene deletions have also been identified, with those having homozygous IGF1 gene deletions exhibiting growth retardation (i.e., short stature), reduced bone size, and reduced vertebral bone mineral density.[12,13] The results of our study are consistent with these previous findings.

Serum OC levels have been widely used as a biomarker of bone turnover and bone formation.[14] Previous studies have evaluated the potential role of OC as a predictor and surrogate marker of fracture or osteoporosis.[15] Studies indicated that bone formation was decreased in patients with IUGR, which was reflected by OC con-

centrations in the cord blood being 20% lower than those of age- or weight-matched newborns.[16] In our study, the OC levels of the IUGR group were lower than those of the control group (P = 0.004).

During the perinatal period, BALP is a reliable marker of bone formation.[17] An increase in liver ALP typically requires increased hepatic lipid production. Protein restriction during pregnancy affects fetal liver lipid metabolism.[18] Lipid metabolism may be influenced by a low protein diet in animal models, which suggests that BALP levels of the IUGR group would be lower than those of the control group. Briana *et al* [13] also reported significant differences in BALP levels between the control and IUGR groups (P < 0.001).

The OPG/RANK/RANKL signaling pathway is a critical regulatory pathway that maintains a balance between the activity of osteoblasts and osteoclasts that prevents bone loss and ensures normal bone turnover.[19] Hossein *et al* [20] demonstrated the positive effects of folic acid supplementation during pregnancy on both maternal and fetal markers of bone turnover (i.e. OPG, RANKL, and OC). This study demonstrated that the RANKL/RANK/OPG system might play an important role in bone formation during pregnancy. Our results indicated that the levels of OPG and the OPG/RANKL ratio of the IUGR group were lower than those of the control group, thus indicating that IUGR affects bone formation. Tenta *et al* [21] also demonstrated that newborns with IUGR exhibited lower RANKL values and higher OPG / RANKL ratios, while newborns that were large for their gestational age (LGA) had higher RANK levels compared with healthy controls (P = 0.036 and 0.044, respectively). This result was similar those obtained in our study.

IUGR infants who receive sufficient nutrition postnatally appear to catch up in growth through accelerated bone formation. In our study, at 3 weeks of age, the serum levels of BALP and OC (P = 0.003 and 0.001, respectively) of the IUGR group were significantly higher than those of the control group. No significant differences were found the in the levels of BALP, OPG, OPG/RANKL, IGF-1, or OC between the two groups at 8 weeks of age (P > 0.05).

The diet of the IUGR group in our study was primarily protein restricted, without any limitations in vitamin, calcium, or phosphorus. Thus, the calcium and phosphorus supply provided in this study should have been sufficient for the rats. Indeed, in our cohort study, serum calcium and phosphorus concentrations were similar between IUGR cases and the control group. In a clinical study, Briana *et al* [13] also revealed that the level of calcium remained unchanged in IUGR newborns.

Puerarin-induced changes in bone growth and turnover markers in IUGR individuals

Puerarin has an analogous structure to that of genistein. Phytoestrogens are plant-derived non-steroidal compounds that bind to estrogen receptors (ERs) and have estrogen-like activities.[22] In recent years, an increasing number of studies have confirmed that puerarin has weak estrogenic activity, including the induction of bone formation and other bone-protective effects; however, its mechanism of action on bone remains unclear. A number of researchers have proposed that puerarin affects bone through ERs, with effects similar to those of 17-β-estradiol. An *et al* [23] proposed that the osteogenic effects of phytoestrogens are based on their ER-mediated and/or enzyme-inhibiting effects. ER-mediated activity was proposed to occur via the preferential binding of phytoestrogens to ER-β, which acts as a dominant-negative regulator of estrogen signaling.[24] However, Liang *et al* [25] suggested that puerarin might play a protective role in diabetic osteoporosis via the reduction of caspase-3 expression.

Previous studies demonstrated that estrogens also play an essential role in pubertal growth via the GH/IGF-I axis.[26] Indeed, estrogens stimulate GH secretion, which induces hepatic synthesis of IGF-I.[27] In our study, after puerarin administration, the level of IGF-1 increased. We propose that puerarin has estrogen-like activity that may stimulate GH secretion, which subsequently increases IGF-1 secretion.

Oral estrogen treatment has been previously associated with weight gain in patients.[28] In our study, after the administration of puerarin, body weight also significantly increased. This effect might be associated with increased hepatic lipid production, which could account for the increased BALP levels in the IUGR group after puerarin treatment.

OC is produced by osteoblasts during bone formation. Circulating OC levels are likely to include molecules derived from bone resorption, when OC embedded in the bone matrix is released.[29] We propose that accelerated bone formation induced by puerarin increased the levels of OC in our study.

The RANKL/RANK/OPG system is regulated by many osteotropic hormones and cytokines. A previous study demonstrated that transforming growth factor-β (TGF-β) and estrogen could activate this system.[30] In addition, Rubin *et al* [31] found that IGF-I increased RANKL expression and decreased OPG expression in mouse stromal cells *in vitro*. In our study, the levels of OPG and the OPG / RANKL ratio increased after puerarin administration. Thus, we propose that puerarin has estrogen-like activity, which stimulates OPG secretion and reduces RANKL secretion.

In this study, we identified a number of bone markers that were affected in IUGR rats. Although the observed changes were small, they were consistent and significant. Future studies are needed to evaluate the mechanism that underlies changes in bone turnover markers during infancy.

Our study demonstrated that rats with IUGR have altered bone metabolism. Puerarin promotes the recovery of bone metabolic markers to normal levels, sug-

gesting that it promotes bone turnover. Further research, particularly large-scale surveys, are needed to investigate the expression of these bone biomarkers in newborns with IUGR and to gain an improved understanding of the mechanism of bone formation.

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