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Markers of bone metabolism, serum leptin levels and bone mineral density in preterm babies

DOI 10.1515/jpem-2013-0474
Received December 8, 2013; accepted June 8, 2015; previously published online July 16, 2015

Abstract: The prospective study assessed the influence of serum leptin levels on markers of bone metabolism and bone mineral density in 2-year-old infants born preterm. A total of 57 randomized preterm Caucasian newborns (32nd–37th week of gestation) were included in the study. Bone metabolism markers were measured every 6 months. The infants were monitored prospectively up to the age of 2 years. When the infants turned 2 years of age, they were investigated by dual energy X-ray absorptiometry (lumbar spine). The median cord blood leptin levels was 3.07 μg/L. The median leptin level during check-ups before 2 years of age was 9.96 μg/L. The other laboratory markers were within the normal ranges for that age. The bone mineral density reached, on average, 0.410 g/cm^2. Lower leptin levels in the cord blood and in the serum of preterm infants do not influence bone mineral density during the first 2 years of life.

Keywords: bone mineral density; calcium; 25-hydroxyvitamin D; leptin; phosphorus; preterm newborn.

Introduction

A premature infant is a baby born before 37 completed weeks of gestation. In almost all countries with reliable data, preterm birth rates are increasing (http://www.who.int/mediacentre/factsheets/fs363/en/). After birth, there are dramatic physiological changes in bone metabolism resulting from various factors: disruption in the maternal mineral supply, stimulation of calciotropic hormone secretion, changes in the hormonal environment, and a relative reduction in mechanical stress. These events stimulate the remodeling process and lead to an increase in endosteal bone resorption and a decrease in bone density. In preterm infants, these adaptation processes modify the mineral requirement, as, by itself, the increased remodeling provides a part of the mineral requirement necessary for postnatal bone growth and development. The care of newborn premature infants should not necessarily aim to achieve intrauterine calcium accretion rates (1).

Thus, two mainstream hypotheses for the role of leptin on bone have emerged: (i) direct regulation through increased osteoblast proliferation and differentiation and (ii) indirect suppression of bone formation through a hypothalamic relay. At the present time, it remains unclear whether these effects are relevant in only extreme circumstances (i.e. models with complete deficiency) or play an important homeostatic role in the regulation of peak bone acquisition and skeletal remodeling. The leptin acts primarily through peripheral pathways and increases osteoblast numbers and activity (2–4).

The classical role of leptin is to provide information about energy stores to the central nervous system and to reduce appetite if the energy stores are full. Maternal and fetal plasma leptin levels are dysregulated in pathological conditions such as gestational diabetes, preeclampsia and intra-uterine growth retardation, representing an effect or a cause of disturbances in the feto/placento/maternal unit. During pregnancy and at birth there is evidence for augmented maternal and fetal leptin levels. This increase is explained in part by leptin production by the placenta. So far, the role of increased leptin production during pregnancy remains unclear. It may be hypothesized that increased leptin levels during pregnancy are part of a teleologically old and redundant system ensuring fetal growth and development, even in periods of reduced maternal energy supply (5). The null hypothesis was that leptin serum level has an influence on bone mineral density (BMD) in preterm newborns.

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Materials and methods

The study was observational. We included moderate (32+0 to 34+0 weeks of gestation) and mild (34+1 to 36+6 weeks of gestation) preterm newborns who were delivered between 32+0 and 36+6 weeks of gestation. The parents agreed to include their children in our study before delivery and a written informed consent for participation was obtained from the mothers. The study received approval from the Research Ethics Committee of the University Hospital in Hradec Králové. The study included only babies whose parents agreed with all study processes.

A total of 57 preterm Caucasian newborns (delivered between 32+0 and 36+6 weeks of gestation) participated. They were divided into two groups: A, born between 32+0 and 34+0 weeks of gestation (n=26); and B, born between 34+1 and 36+6 weeks of gestation (n=31). There were 28 boys and 29 girls included in the study. There were 14 boys and 12 girls in group A and 14 boys and 17 girls in group B.

Mothers: 17 mothers received glucocorticoid before delivery, four mothers had gestational diabetes (three were treated by diet, one received insulin therapy), eight mothers were treated with levothyroxine.

The type and time of delivery was recorded. None of the subjects had any postnatal complications. The length of the cord (the baby's portion and the portion from the placenta) was registered along with the placenta's weight and diameter. The length and weight of the newborns were measured using certified equipment and a calibrated weight scale. Cord blood samples were collected and the pH was measured. Then the serum was separated by centrifugation and frozen at –70°C for further analysis of leptin, osteocalcin, calcium, phosphate, and 25-hydroxyvitamin D (25-OH D).

The osteocalcin measurements were performed using monoclonal mouse antibodies against N-MID OC using electrochemiluminescence with an analyzer (Elecsys N-Mid Osteocalcin, Roche Diagnostics, Mannheim, Germany). The coefficient of variation in this method is ≤6.5% (6). Leptin measurements were performed using polyclonal antihuman leptin antibodies conjugated with horseradish peroxidase (HRP) by the enzyme linked immuno sorbent assay (ELISA, BioVendor, Brno, Czech republic) method using an absorbance measure (which is proportional to the concentration of leptin) with a 650 nm microplate reader filter. The coefficient of variation in this method is ≤7.6% (7).

The levels of 25-OH D were measured by a LIAISON 25 OH Vitamin D TOTAL Assay (310600) – chemiluminiscent immunoassay (CLIA) technology using an analyzer (Liaison Light Check, DiaSorin, Stillwater, MN, USA). The coefficient of variation in this method is ≤5.5% (8).

The levels of calcium (Ca) and phosphorus (P) were measured by photometric analyses using a Modular PP analyzer (Hitachi, Hitachi, Japan). The levels of ionised calcium (iCa) were measured by an ion-selective electrode using a Nova 8 analyzer (Nova Biomedical, Waltham, MA, USA). The levels of alkaline phosphatase (ALP) were measured with a 4-nitrophenyl phosphate substrate (Sigma-Aldrich, St. Louis, MO, USA).

The babies were monitored regularly over the 2-year period. The serum levels of leptin, osteocalcin, total and ionized calcium, phosphorus, ALP, alanine aminotransferase, and 25-OH D were measured in venous blood every 6 months. A lumbar spine (L1–L4) BMD investigation was performed at 2 years of age using dual energy X-ray absorptiometry (Hologic, Bedford, MA, USA). This investigation insisted 20 s so the children tolerated it well. Relevant past medical history of the mothers which could have had an influence on leptin levels was considered – disorders such as hypothyroidism, bronchial asthma, ulcerative colitis, multiple sclerosis, arterial hypertension, diabetes mellitus, pulmonary embolism; medication such as corticosteroids, levothyroxin, antihistaminic drugs, beta-blockers, anticoagulants, vitamins and minerals; weight gain during gravidity; oral glucose tolerance test results; and smoking during pregnancy.

Results

Anthropometric data: Four children (7%) were evaluated as hypotrophic (birth weight under the 3rd percentile for gestational age and gender). Seven newborns (12.3%) were under the 10th percentile. Seven children (12.3%) were estimated as hypertrophic (birth weight above the 90th percentile for gestational age and gender). The Rohrer's ponderal index (calculated as weight/length^3) has a range of 13.9–31.4, with a median of 24.6.

Laboratory markers: The median of the cord blood leptin levels was 3.07 μg/L (min. 1.0; max. 40.2). The median of leptin levels (cord blood) in group A was 2.89 μg/L (min. 1.21; max. 36.3). The median of leptin levels (cord blood) in group B was 3.13 μg/L (min. 1.0; max. 40.2). The median of the serum leptin level during check-ups before the age of 2 years was 9.96 μg/L (min. 0.13; max. 19.8). It was 1.40 μg/L (min. 0.36, max. 5.69) in group A and 1.67 μg/L (min. 0.35; max. 19.8) in group B. Results from controls are presented in Table 1. Lumbar spine (L1–L4) BMD at the age of 2 years (±2 months) ranged from 0.340 to 0.502 g/cm^2. The correlation between BMD and leptin levels did not reach statistical significance ($p=0.711$; $r^2=0.0067$; β coefficient=0.0003). No statistical significance was found in relation to lumbar spine BMD at 2 years of age nor in relation to type of delivery ($p=0.16$), the mother smoking ($p=0.295$), the weight of the placenta or the cord length ($p=0.33$).

We used the significance level $\alpha=0.05$ and present the p value. A two-sample t-test was used to compare the type of delivery (caesarean section, vaginal delivery) and BMD ($p=0.16$). The same test was used to compare smoking during pregnancy and BMD ($p=0.30$). We used multiple regression analysis to see if there was a dependence of BMD on cord length ($p=0.33$), on birth weight ($p=0.77$), on weight gain during pregnancy ($p=0.59$), on placenta diameter ($p=0.22$), and on serum leptin levels in cord blood ($p=0.22$). Here, a p value higher than the significance level means we accept their dependence.

We perform Pearson correlation tests between baseline leptin levels with other quantitative variables

Table 1: Average of analyses during check-ups in both groups of newborns.

Check-ups	Ca, mmol/L	iCa, mmol/L	P, mmol/L	ALP, µkat/L	Leptin, µg/L	Osteocalcin, µg/L	Vitamin 25-OH D, nmol/L
At the age of 6 months							
Group A	2.55	1.52	1.88	4.44	2.51	103	126
Group B	2.52	1.38	1.85	4.62	2.29	89.6	108
At the age of 12 months							
Group A	2.56	1.4	1.86	4.46	2.5	107	125
Group B	2.5	1.36	1.77	3.93	1.59	78.5	72.6
At the age of 18 months							
Group A	2.44	1.3	1.64	4.14	1.13	118	111
Group B	2.47	1.34	1.68	4.12	3.02	78.7	72.4
At the age of 24 months							
Group A	2.46	1.34	1.76	4.03	1.2	81	64.1
Group B	2.2	1.35	1.78	3.92	1.58	79.6	71.2

This table shows a comparison of laboratory analysis results between group A (born between 32+0 and 34+0 weeks of gestation) and group B (born between 34+1 and 36+6 weeks of gestation).

Table 2: Pearson correlation tests between baseline leptin levels with other quantitative variables.

Analyses	r	p-Value
Group A		
Leptin		
Ca	0.38	0.000
iCa	0.42	0.000
P	−0.25	0.010
ALP	−0.10	0.340
Osteocalcin	0.04	0.087
25-OH D	0.10	0.320
Group B		
Leptin		
Ca	0.10	0.320
iCa	0.02	0.860
P	−0.01	0.930
ALP	0.00	1.000
Osteocalcin	−0.01	0.940
25-OH D	0.08	0.410

This table shows correlation between baseline leptin levels with other quantitative variables.

(Table 2). We used paired t-test for intragroup comparisons of following variables (bone markers, leptin/weight ratio, 25-OH D, calcium, and phosphate; Table 3). We used one-side alternative. We asked if the values are greater than the upper limit of the interval of the physiological values. Results and physiological values are in Tables 4 and 5.

We used nonparametric Mann-Whitney test for comparing serum leptin levels in the cord blood in the group of mothers without therapy during pregnancy and mothers using levothyroxine (p=0.046). We used same test for comparing serum leptin levels in the cord blood in the group of mothers using levothyroxine and glucocorticoids (p=0.127).

Table 3: Paired t-test – intragroup comparisons.

Analytes	p-Value
Paired t-tests – Group A	
Leptin/weight	
ALP	0
Osteocalcin	0
25-OH D	0
Ca	0
iCa	1
P	0.71
25-OH D	
ALP	1
Osteocalcin	1
Ca	1
iCa	1
P	1
Osteocalcin	
ALP	1
Ca	1
iCa	1
P	1
Paired t-tests – Group B	
Leptin/weight	
ALP	0.98
Osteocalcin	1
25OHD	0
Ca	0.17
iCa	1
P	1
25OH D	
ALP	1
Osteocalcin	0.88
Ca	0
iCa	1
P	1
Osteocalcin	
ALP	1
Ca	1
iCa	1
P	1

Table 4: Independent samples t-test.

	Mean	SD	μ	p-Value
Group A				
Ca	2.53	0.01	2.40	0.00
iCa	1.33	0.12	1.15	0.00
P	1.90	0.34	1.90	0.56
Leptin	3.05	5.33	3.50	0.82
Osteocalcin	60.50	24.80	30.00	0.00
25-OH D	102.00	60.20	47.50	0.00
ALP	4.22	1.03	9.00	1.00
Group B				
Ca	2.44	0.25	2.40	0.02
iCa	1.29	0.32	1.15	0.00
P	1.93	0.51	1.90	0.22
Leptin	3.27	4.90	3.50	0.70
Osteocalcin	71.00	53.50	30.00	0.00
25-OH D	87.40	46.10	47.50	0.00
ALP	5.09	5.61	9.00	1.00

This table compares intragoups results.

Table 5: Reference values of laboratory parameters.

Calcium	Newborn	1.9–2.8 mmol/L
	Suckling+toddler	2.0–2.9 mmol/L
Ionized calcium	Newborn	1.0–1.15 mol/L
	Suckling+toddler	1.1–1.3 mmol/L
Phosphor	Newborn	1.5–2.5 mmol/L
	Suckling+toddler	1.3–2.3 mmol/L
25-OH D		20–75 ng/mL
ALP	0–2 years	6–12 μkat/L
Osteocalcin		10–50 ng/mL

During the study we lost five patients. One patient from group A and four patients from group B. This was parents' decision. Patients from group B had less count of blood check-ups (planned by their physician) compared to patients from group A.

Discussion

In the group of babies born between the 32nd and 34th weeks of gestation the leptin level was lower in the cord blood, which is in agreement with the literature. There are many factors influencing BMD during early infancy. In this prospective study, we investigated the role of leptin serum concentrations during early postnatal life in preterm newborns. Cord serum leptin levels are usually higher in girls than in boys. Gender, gestational length, and maternal smoking are reported as important factors associated with umbilical cord serum leptin concentration in newborns (9). The serum leptin levels in the cord blood could be influenced by the therapy during pregnancy. The therapy affects the babies' metabolism too.

We do not reject the null hypothesis (H0: Independence) in the correlation between leptin and ALP, 25-OH D and osteocalcin in the group A. We accept the null hypothesis in correlation in group B. There should be sign of regulations immature in group A.

A premature infant may have signs of the following problems which may lead to lower BMD in the future: less body fat, lower muscle tone, and less activity than full-term infants, problems feeding due to trouble sucking or coordinating swallowing and breathing (http://www.nlm.nih.gov/medlineplus/ency/article/001562.htm).

The effect of leptin on bone is controversial. Although in vitro studies have shown that leptin stimulates osteoblast differentiation and mineralization and inhibits osteoclastogenesis, some rodent studies have shown that leptin administered centrally might result in decreased bone formation. Leptin given at very high doses maintains BMD, microarchitecture, and mechanical strength in female rats, despite a significant decrease in body weight (10). Thus, two mainstream hypotheses for the role of leptin on bone emerge: (i) direct regulation through increased osteoblast proliferation and differentiation and (ii) indirect suppression of bone formation through a hypothalamic relay. Visceral fat and the leptin/adiponectin ratio were negative predictors for BMD and content in obese adolescent girls (11).

At the present time, it remains unclear whether these effects are relevant in only extreme circumstances (i.e. models with complete deficiency) or if they play an important homeostatic role in the regulation of peak bone acquisition and skeletal remodeling (3). Leptin acts primarily through peripheral pathways and increases osteoblast numbers and activity (4). Studies have shown that leptin levels are lower in preterm newborns. There is a connection between birth weight and the leptin levels in serum (the lower the birth weight, the lower the leptin level). Higher leptin levels can be found in babies with diabetic mothers. There is increased accumulation of adipose tissue, which is characteristic for such infants (12, 13). The serum leptin level reveals that the level of nutrition is equal to the level of hormones. In preterm male infants, serum leptin concentration increases with postnatal weight and testosterone may suppress leptin synthesis (13). In vitro studies have shown that the leptin levels are induced in the syncytiotrophoblast by corticotropin-releasing hormones. This process might promote the fetal nutrient supply and placental corticosteroid metabolism in the period before labor induction (14). Leptin stimulates linear growth by regulating the energy balance of the

organism and by stimulating the production and secretion of growth hormone from the hypothalamus; at the same time, it is involved with bone remodeling and has a direct effect on the chondrocytes of the growth plate (15, 16). The circulating leptin concentration increases markedly after 34 weeks of gestation and bears a close temporal relation with the exponential accumulation of body fat mass during that period (17). Maternal leptin serum concentrations at the time of amniocentesis correlate significantly with BMI with maternal skinfold measurements, but leptin levels in amniotic fluid did not correlate with maternal BMI and skinfold thickness (18). Placental weight correlated inversely with leptin levels in maternal serum at birth. Leptin concentrations in velus cord blood correlated significantly with the levels in arterial cord blood and leptin levels in cord blood correlated positively with birth weight (19). The rapid decline in the circulating concentrations of leptin after birth may be of physiological advantage to preterm and term newborns by limiting their body energy expenditure and conserving nutritional reverses for subsequent growth and development (17, 20). This may be important for the stimulation of feeding behavior and the acquisition of energy homeostasis (20, 21).

There are factors which increase leptin levels (e.g. sex steroids, fat, insulin, estrogen, proinflammatory cytokines) and factors which reduce leptin levels (e.g. decreasing fat stores, fasting, thyroid hormone, testosterone, adrenergic agonists, testosterone) (22). Our study included children whose mothers used glucocorticoids and levothyroxin during pregnancy.

An animal study (rat models) has revealed that total bone length and mineral density as well as tibial growth-plate width and the numbers of cells within its zones were significantly greater in offspring treated with leptin than in the control group (23). The results from a clinical study with adult patients with weight loss suggest that both decreased bone formation and increased bone resorption underlie bone loss associated with weight loss. Leptin administration did not prevent the uncoupling of bone remodeling that accompanies weight loss (24).

It is known that lower bone density should be due to a homozygous mutation in pre-miR-2861 that blocks expression of miR-2861. This state causes primary osteoporosis (25).

Our results support the hypothesis that the serum leptin level is not a significant determining factor in skeletal development during early infancy. Some possible areas of future research could focus on observing changes in leptins levels in very low birth weight newborns during early infancy and a possible connection with BMD and markers of bone metabolism in babies whose mothers suffer from hypothyroidism. Check-ups saw no significant differences between the two groups (group A and group B) of preterm newborns.

Study limitations

None of the included mothers had any clinical signs of vitamin D deficiency but their serum 25-OH D was not measured. Only four (7%) hypotrophic newborns were included. Participating children had no postnatal complications, making the parents perhaps less motivated to complete this study, while a number of parents simply refused to have their children's BMD measured at the end of study.

Summary

Leptin plays an important role in the regulation of bone metabolism. It directly influences osteoblast proliferation and differentiation. Indirectly, leptin affects bone formation through a hypothalamic relay. The leptin level in the cord blood of preterm newborns is significantly lower than the leptin level in the cord blood of term delivered newborns. However, leptin levels in the cord blood in newborns delivered between 32+0 and 36+6 weeks of gestation do not significantly influence the lumbar spine BMD at 2 years of age.

Acknowledgments: The authors are grateful to Renfro MS, BA, for assistance with the language correction of the manuscript; Kubečková A, MD, for assistance with the patients' inclusion into the study and J. Bukač, MSc, PhD, for assistance with statistical analysis.

Funding: The study was supported by Grant Agency of Charles University (GAUK) in Prague project No 432411 and financial support was provided by the Medical Faculty in Hradec Králové, Charles University in Prague.

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