Vitamin D Deficiency in Guinea Pigs: Exacerbation of Bone Phenotype During Pregnancy and Disturbed Fetal Mineralization, with Recovery by 1,25(OH)₂D₃ Infusion or Dietary Calcium-Phosphate Supplementation

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Abstract. Vitamin D (D) deficiency during human pregnancy appears to disturb fetal growth and mineralization, but fetal development is normal in D-deficient rats and vitamin D receptor gene-ablated mice. We used the guinea pig model to investigate maternal and fetal effects of D deficiency. Pregnant (Pr) and nonpregnant (NPr) animals were fed a D-replete (+D) or D-deficient diet (–D) for 8 weeks. We further studied whether the effects of a –D diet are reversed by continuous 1,25(OH)₂D₃ infusion (–D+1,25) and/or by a lactose-, Ca- and P-enriched D-deficient diet (–D+Ca/P). Bone analyses included histomorphometry of the proximal tibiae, dual-energy X-ray absorptiometry (DXA), and quantitative computed tomography (QCT) of the femora. Depletion of 25(OH)D₃ and 1,25(OH)₂D₃ levels and the D-deficiency syndrome were more severe in pregnant animals. Indeed, Pr/–D but not NPr/–D guinea pigs were hypophosphatemic, and showed robust increases in growth plate width and osteoid surface and thickness; in addition, bone mineral density on DXA was lower in Pr/–D animals only, which was exclusively in cortical bone on QCT. Bone phenotype was partly normalized in Pr/–D+1,25 and Pr/–D+Ca/P animals. Compared with +D fetuses, –D fetuses had very low or undetectable 25(OH)D₃ and 1,25(OH)₂D₃, were hypercalcemic and hypophosphatemic, and had lower osteocalcin levels. In addition, body weight and total body bone mineral content were 10–15% lower; histomorphometry showed hypertrophic chondrocyte zone expansion and hyperosteoroidosis. 1,25(OH)₂D₃ levels were restored in –D+1,25 fetuses, and the phenotype was partially corrected. Similarly, the fetal +D phenotype was rescued in large part in –D+Ca/P fetuses, despite undetectable circulating 25(OH)D₃ and 1,25(OH)₂D₃. We conclude that pregnancy markedly exacerbates D

deficiency, and that augmenting Ca and P intake overrides the deleterious effects of D deficiency on fetal development.

Key words: Vitamin D deficiency — Guinea pig — Pregnancy — Fetal mineralization — Histomorphometry.

D deficiency is common in women during the reproductive period and pregnancy. A report of a high prevalence of D deficiency in pregnant Asian women living in Britain [1] has been followed by the recognition that D deficiency is also common in women of reproductive age in more southern regions with abundant sunlight [2–5]. Avoidance of ultraviolet (UV) light exposure — by wearing traditional dress and staying indoors — appears to be the primary risk factor [4, 5], with lower dietary D intake as a secondary factor [5, 6].

In humans, there is fragmentary evidence that D deficiency disturbs fetal development. Cases of ‘fetal rickets’ have been reported in severely D-deficient pregnant women [7]. Babies of D-deficient mothers were found to be more frequently small-for-gestational age [1], and their birth length was correlated with maternal ionized calcium (Ca²⁺) levels [8]. Vitamin D supplements during the third trimester of pregnancy increased birth weight in Asian women [9]. Also, cord plasma alkaline phosphatase levels were raised in D-deficient pregnancies, the newborns had a larger anterior fontanelle, and symptomatic early neonatal hypocalcemia was more common [1]. Furthermore, total-body bone mineral content (BMC) was 6% lower in winter-born than in summer-born newborns in South Korea, and was correlated with cord serum 25-hydroxyvitamin D₃ [25(OH)D₃] levels [10].

Studies in small rodents failed to show an effect of D deficiency on fetal growth and mineralization. In D-deficient rats, fetal body weight and Ca content/

weight [11], trabecular bone volume on tibial histomorphometry [12] and plasma Ca and phosphate (P) levels [13] were found to be normal, although there was a significant increase in osteoid area [12]. In fact, when corrected for lower milk intake, D deficiency did not affect growth and mineralization until day 20 of postnatal life [14]. Similarly, growth, bone histology, and plasma Ca and parathyroid hormone (PTH) levels were normal before postnatal day 21 in mice with a vitamin D receptor (VDR) gene knockout; however, an expansion in the hypertrophic chondrocyte width was observed by day 15 [15, 16].

Fetuses of small rodents are born chemically and physiologically immature, and growth and bone formation increase rapidly during the suckling and early postweaning stages [17]. In contrast, guinea pig fetuses (gestation length 63–68 days) are mineralized comparably to human fetuses by the end of gestation, and show active bone formation [18]. Herein we examined the effects of a D-deficient diet in guinea pigs on maternal and fetal bone and mineral metabolism. The diet was started on the first day of pregnancy to avoid infertility [19]; we also studied nonpregnant guinea pigs fed the diet during a similar period of 8 weeks. Also, we studied the effect of maternal treatment with 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the only vitamin D metabolite required to prevent rickets in postnatal rats fed a D-deficient diet [20]. We used a dose (5 ng/100 g/day, s.c.) that was shown to maximally prevent rickets in D-deficient rats; indeed, at higher doses, 1,25(OH)₂D₃ may induce, rather than reverse, osteomalacia [21]. 1,25(OH)₂D₃ crosses the placental barrier in humans [22], and we have reported a highly significant correlation between maternal and fetal 1,25(OH)₂D₃ levels in humans [23], guinea pigs [24], and rats [25]. 1,25(OH)₂D₃ treatment was given during the last 4 weeks of pregnancy only, since ossification centers are first detected from fetal day 26 in guinea pigs [26]. Finally, we studied the effect of a high lactose, Ca- and P-enriched D-deficient diet. A comparable diet reduced or even normalized the secondary hyperparathyroidism and the skeletal abnormalities (rickets and osteomalacia) in weaned nutritionally D-deficient rats [27, 28] as well as in weaned mice with an ablated VDR gene [29, 30].

Materials and Methods

Animals

All experiments were approved by the ethical committee for animal research at the K.U. Leuven. Two- to three-month-old (young adult) female guinea pigs, bred at the K.U. Leuven Proefdierencentrum, were housed in a room with a constant temperature of 22°C and a 12 hour light/12 hour dark cycle. All animals were fed a standard guinea-pig chow *ad libitum*, containing 0.96% Ca, 0.49% P, 2400 IU vitamin D₃/kg, and 1600 mg vitamin C/kg (Hope Farms, Woerden, the Netherlands). The animals received deionized water with added vitamin C (400 mg/L), as guinea pigs are susceptible to de-

velopment of vitamin C deficiency. Part of the animals were mated during the day; the presence of a copulation plug was checked twice daily on those days. Once a vaginal plug was detected, the animal was kept in a separate cage and labeled; this day was defined as day 1 of pregnancy. The pregnant animals were divided into four experimental groups: (1) a D-replete group (+D group), which continued on the standard guinea pig diet; (2) a D-deficient group (–D group), fed an experimental diet containing 0.97% Ca, 0.38% P, no vitamin D₃, and 2300 mg vitamin C/kg (Harlan/Teklad, Zeist, the Netherlands); (3) a D-deficient group, which were infused s.c. with 1,25(OH)₂D₃ (0.12 nmol/day) via an Alzet miniosmotic pump (model 2ML4, Charles River, Palo Alto, CA) placed in the back region under anesthesia (ketamine hydrochloride, 48 mg/kg, and xylazine hydrochloride, 6 mg/kg, both i.m.) from day 30 of pregnancy onward (–D + 1,25 group); (4) a D-deficient group fed an experimental diet containing 20% lactose as carbohydrate, 1.9% Ca, 1.0% P, and 2300 mg vitamin C/kg (Harlan/Teklad) (–D + Ca/P group). All animals in groups 2–4 were housed in a UV light-free room. The experimental diets in groups 2–4 were introduced over a 7-day period, in a 50% standard–50% experimental diet mixture, to avoid food deprivation; the experimental diet was then continued for 7 weeks. Nonpregnant animals received the experimental diets for the same duration. All animals received fluorochrome labeling with calcein (Sigma, St. Louis, MO), 16 mg/kg i.m., 8 days and 1 day (24 hours) prior to the end of the study.

Eight weeks after the start of the experiment (day 57 for pregnant animals), the animals were transported to the UV light-deprived surgical room, and were anesthetized as described. The anesthetized animals were weighed, and xylocaine 2% was injected s.c. in the midline abdominal area, prior to laparotomy. In pregnant animals, the uterus was exposed, and amniotic fluid was collected from the gestational sacs and pooled. The fetuses were exposed through a small uterine incision from left to right, and a midline laparotomy was performed in each fetus to obtain a blood sample from the abdominal aorta, with the umbilical cord still attached to the placenta. All fetuses and placentas were weighed. The sample from the last exposed fetus was kept for measurement of Ca²⁺, total Ca, and P; the previous samples were kept for measurement of 25(OH)D₃, 1,25(OH)₂D₃, and osteocalcin. The last two fetuses (or last fetus in case there were only two fetuses per mother) were eviscerated, weighed again, and stored at –20°C for measurement of whole body BMC. The tibiae were removed from the other fetuses for histomorphometry. A blood sample was taken from the abdominal aorta of the mother, and the animal was euthanized by cervical dislocation. Both tibiae were removed for histomorphometry and both femora were removed for measurement of weight and dual-energy X-ray absorptiometry (DXA) and peripheral quantitative computed tomography (PQCT) analysis. The surgical procedure did not exceed 45 min; the Ca²⁺ measurements were done within 10 min of the maternal sample collection.

Assays

All assays, except Ca²⁺ and 1,25(OH)₂D₃, were done in a single run. Ca²⁺ was measured in whole blood by the ion selective electrode method on the Ciba Corning 288 Blood Gas System (Bayer Diagnostics, Brussels, Belgium); plasma P was measured by colorimetry [31]. Plasma 25(OH)D₃ was measured by direct competitive protein binding assay [32]; the detection limit of this assay is about 2.5 µg/L (6.25 nmol/L). Plasma 1,25(OH)₂D₃ was measured by radioimmunoassay (RIA) after HPLC purification, as described [23], with later modifications [31]; the detection limit is around 18 pg for 1 mL plasma (43 pmol/L). Vitamin D-binding protein (DBP) was measured by a single radial immunodiffusion assay specific for guinea pig DBP [18]; the free 1,25(OH)₂D₃ index was calculated as the 1,25(OH)₂D₃/DBP molar index (×10^{–5}) [23]. Plasma osteocalcin was measured by RIA with guinea pig osteocalcin as the standard and a rabbit polyclonal antiserum

[18]. Amniotic fluid deoxypyridinoline, which was used as a measure of fetal bone resorption, was measured fluorometrically after acid hydrolysis, extraction, and HPLC separation, as described previously; values were corrected for creatinine concentrations [33].

Bone Studies

Wet and dry weights were obtained for all femora; to obtain dry weights, bones were dried in ethanol and diethylether (1/1, vol/vol) and then diethylether.

DXA was performed on both adult femora and on whole fetuses using a Hologic QDR-1000 absorptiometer (Waltham, MA), as described previously [24, 34]. For the adult femora, we measured the BMC and bone mineral density (BMD) of the whole femur, of a mid-diaphyseal area consisting exclusively of cortical bone, and of a metaphyseal area at 20% from the distal end of the femur consisting of both trabecular and cortical bone. The coefficient of variation (CV) of 8 consecutive measurements of adult femora was 0.8% for the whole femur BMD, 1.7% for the mid-diaphyseal BMD, and 2.1% for the metaphyseal BMD. The CV of 10 consecutive measurements of whole fetus BMC was 4.7%.

PQCT was performed on adult and fetal femora using a XCT Bone Scanner (Norland, Fort Atkinson, WI) and software 5.40. Slices were 0.3 mm thick, and voxel size was 0.07 mm. Trabecular bone density was measured in an inner area of 25% of the total cross-sectional area, at 3, 4, and 5 mm from the distal growth plate in the adult femora, and at 4.25, 4.5, and 4.75 mm from the distal end of the femur in the fetuses. Cortical bone density was measured at 15 mm from the distal growth plate in the adult femora, and at 10 mm from the distal end of the femora in the fetuses. Between-measurement CV was <1%.

Histomorphometry of adult and fetal proximal tibiae was performed as described previously [24, 34], using the nomenclature proposed by Parfitt et al. [35]. The undecalcified left proximal tibia was embedded in methylmetacrylate, and 4 μ m-thick longitudinal sections were cut. Three sections were measured per animal, with 800 μ m in between. Static and dynamic morphometry were performed using a VIDAS 21 Image Analyzing Computer (Kontron, Munich, Germany) or a KS 100 Image Analyzing Computer (Carl Zeiss, Hallbergmoos, Germany). We used von Kossa-stained sections for the measurement of trabecular bone volume (BV/TV), sections stained with a modified Goldner-trichrome for the measurement of osteoid surface (OS/BS), and osteoid thickness and the growth plate width, and unstained sections for the fluoromicroscopic measurement of double- and single-labeled surfaces (dLS/BS and sLS/BS, respectively), and the mineral apposition rate (MAR). In adult tibiae, growth plate width was measured over the full length of the growth plate at $\times 150$ magnification. BV/TV was measured in 4 consecutive fields in the vertical axis of the central metaphysis at $\times 150$ magnification, excluding the primary spongiosa (i.e., starting at 1 mm from the distal growth plate). The other measurements were performed in the secondary spongiosa, in as many fields as possible, excluding the endocortical and subcortical bone surfaces: 37 ± 1 fields (mean $\pm$ SEM, $n = 180$) were assessed per section, corresponding to $38,960 \pm 1,197 \mu$ m trabecular bone surface. For dynamic morphometry, we measured 101 ± 14 double labels per section; the MAR was measured at 3 equidistant points per double label. The mineral formation rate, surface-referent (MFR/BS), was calculated as the mineralizing surface (MS/BS = dLS/BS + 1/2 sLS/BS) * MAR * $\pi/4$. In fetal tibiae, growth plate width could not be measured because there was a variable degree of growth plate ossification. We therefore measured the area occupied by hypertrophic chondrocytes relative to the total chondrocyte area of the growth plate (calculated as a %), at $\times 75$ magnification. Dynamic morphometry could not be performed in sections from fetal tibiae because the fluorochrome labeling—though abundant—was patchy. OS/BS and O.Th. at the trabecular bone surface were measured at $\times 300$

Fig. 1. Plasma concentrations of 25(OH)D₃ and 1,25(OH)₂D₃ in nonpregnant, pregnant (day 57), and fetal guinea pigs. Groups: vitamin D-replete (+D), black bars, vitamin D-deficient (-D), open bars, vitamin D-deficient with 1,25(OH)₂D₃ treatment (-D+1,25), squared bars; vitamin D-deficient with lactose-, Ca-, and P-enriched diet (-D+Ca/P), hatched bars. The number of data (n) and the number of undetectable values (n-) are shown. 25(OH)D₃ levels were undetectable below 2.5 ng/mL (6.25 nmol/L), and 1,25(OH)₂D₃ levels were undetectable below 18 pg/mL (43 pmol/L); for data analysis, samples with undetectable 25(OH)D₃ and 1,25(OH)₂D₃ were given a value of 1.25 ng/mL and 9 pg/mL, respectively. Statistical analysis: **a**, significant difference with the respective (nonpregnant, pregnant, or fetal) +D group ($P < 0.01$); **b**, significant difference with the respective -D group ($P < 0.05$); **bb**, $P < 0.01$); **c**, significant difference between pregnant vs. nonpregnant animals of the same diet group ($P < 0.05$); **cc**, $P < 0.01$); **d**, significant difference between fetal vs. maternal values of the same diet group ($P < 0.05$); **dd**, $P < 0.01$).

magnification, in 11 ± 0.3 fields per section, corresponding to $51,436 \pm 2,689 \mu$ m bone surface ($n = 186$).

The right tibia of adult animals was also processed for measurement of the tartrate-resistant acid phosphatase (TRAP)-positive bone surface. Tibiae were decalcified, embedded in methylmetacrylate and JB-4 kit (Polysciences, Warrington, PA) in a 1:3 ratio, and 4 μ m sections were obtained. TRAP staining was performed as described previously [34]. Measurements were performed in the metaphyseal secondary spongiosa as for OS/BS; the surface covered by TRAP-positive cells was denoted as TRAPS/BS.

Data Analysis

A software program was used (NCSS, Kaysville, UT). Adult (both nonpregnant and pregnant) and fetal data were first

Table 1. Body weight, blood Ca²⁺, plasma phosphate (P), and osteocalcin (OC) levels in nonpregnant, pregnant, and fetal guinea-pigs

	+D	−D	−D+1,25	−D+Ca/P	One-way ANOVA
Body weight (g)					
Nonpregnant	762 (40)	922 ^a (42)	820 (49)	780 ^b (40)	$P < 0.001$
Pregnant	1168 ^{cc} (39)	1073 ^c (49)	1100 ^{cc} (56)	1152 ^{cc} (24)	
Fetuses	57.0 (1.1)	50.1 ^{aa} (1.1)	47.8 ^{aa} (1.6)	54.5 ^{bb} (1.0)	$P < 0.001$
Ca ²⁺ (nmol/l)					
Nonpregnant	1.39 (0.03)	1.36 (0.03)	1.42 (0.02)	1.34 (0.03)	$P = 0.006$
Pregnant	1.31 (0.04)	1.31 (0.03)	1.19 ^{abcc} (0.02)	1.35 (0.04)	
Fetuses	1.52 ^{dd} (0.04)	2.01 ^{aadd} (0.22)	1.33 ^{bdd} (0.05)	1.54 ^b (0.13)	$P = 0.01$
P (mmol/l)					
Nonpregnant	1.65 (0.02)	1.62 (0.04)	1.69 (0.02)	1.76 ^{abb} (0.04)	$P < 0.001$
Pregnant	1.68 (0.02)	1.33 ^{aacc} (0.01)	1.58 ^{abbc} (0.06)	1.57 ^{aabbc} (0.03)	
Fetuses	1.89 ^{dd} (0.04)	1.71 ^{aadd} (0.03)	1.77 (0.10)	1.89 ^{bdd} (0.02)	$P = 0.02$
OC (nmol/l)					
Nonpregnant	7.3 (0.5)	7.8 (0.6)	9.7 ^a (0.6)	8.6 (0.6)	$P = 0.04$
Pregnant	7.4 (0.4)	9.0 (0.6)	9.2 (1.3)	7.2 ^b (0.6)	
Fetuses	62 ^{dd} (3)	48 ^{aadd} (3)	62 ^{bdd} (4)	61 ^{bdd} (3)	$P < 0.001$

Number of data in each group as in Fig. 1 Statistical analysis: one-way ANOVA was carried out for adult (nonpregnant and pregnant) and for fetal guinea pigs separately, followed by Fisher’s LSD multiple comparison test
^aSignificant difference with the respective (nonpregnant, pregnant or fetal) +D group (^a $P < 0.05$, ^{aa} $P < 0.01$); ^b Significant difference with the respective (nonpregnant, pregnant or fetal) −D group (^b $P < 0.05$, ^{bb} $P < 0.01$); ^c Significant difference between pregnant group and the respective nonpregnant group (^c $P < 0.05$, ^{cc} $P < 0.01$). Maternal-fetal differences for Ca²⁺, P and osteocalcin were examined by paired *t* tests: ^d Significant difference between the fetuses and their mothers in the same group (^d $P < 0.05$, ^{dd} $P < 0.01$)

analyzed separately. One-way ANOVA was performed for each variable; if $P < 0.05$, Fisher’s LSD multiple comparison test was used to detect significant differences between individual groups at the $P < 0.05$ and $P < 0.01$ level. Blood/plasma data of mothers and fetuses belonging to the same diet group were compared with paired *t* tests.

Results

Adult Guinea Pigs

Plasma 25(OH)D₃ concentrations were lower in pregnant than in nonpregnant +D guinea pigs (Fig. 1), confirming our previous data [18, 24]. 25(OH)D₃ was low or undetectable in guinea pigs fed the D-deficient diet (−D, −D+1,25, and −D+Ca/P groups); levels were more often undetectable in pregnant (14/23) than in nonpregnant (2/20) animals of these three groups. Plasma 1,25(OH)₂D₃ levels were significantly lower in nonpregnant and pregnant −D guinea pigs, compared with their respective +D-groups. 1,25(OH)₂D₃ treatment in −D animals restored 1,25(OH)₂D₃ toward levels comparable to (nonpregnant) or somewhat above (pregnant) those of +D animals. In contrast, Ca/P supplementation further reduced 1,25(OH)₂D₃ levels in nonpregnant −D animals. During pregnancy, 1,25(OH)₂D₃ levels were raised in +D, −D+1,25, and −D+Ca/P, but not in −D, guinea pigs. DBP levels were lower in the pregnant than in the respective nonpregnant groups (data not shown), confirming our previous data [18, 24]. Changes in the free 1,25(OH)₂D₃ index between

groups mirrored those of total 1,25(OH)₂D₃ levels (data not shown).

Body weight varied little: nonpregnant −D animals weighed more than +D and −D+Ca/P animals, but there was no such effect in pregnant guinea pigs (Table 1). Ca²⁺ levels were unchanged in −D animals, but pregnant guinea pigs in the −D+1,25 group had lower Ca²⁺ levels than +D or −D animals. Plasma *P* levels were decreased in pregnant, but not nonpregnant −D guinea pigs compared with the respective +D groups. *P* levels were partly restored by both 1,25(OH)₂D₃ treatment and Ca/P supplementation. Pearson correlation analysis of 25(OH)D₃, 1,25(OH)₂D₃, Ca²⁺, and *P* levels showed that plasma 25(OH)D₃ and *P* levels were correlated ($r = 0.58$, $P < 0.001$, $n = 61$). There was no effect of a D-deficient diet on plasma osteocalcin concentrations.

Wet and dry weight of the femora were not different among groups (ANOVA: $P = 0.39$ and $P = 0.17$, respectively). On DXA analysis, the BMD of the left femur was lower in pregnant −D guinea-pigs, compared with pregnant +D and nonpregnant −D animals, and was normalized by Ca/P supplementation (Fig. 2). Similar results were obtained for the right femur (ANOVA: $P = 0.003$; data not shown). There was no significant difference in the BMD measured at a mid-diaphyseal area of the femur (ANOVA: $P = 0.08$ and $P = 0.18$ for the left and right femur, respectively), and in the BMD at a distal metaphyseal area ($P = 0.08$ and $P = 0.06$, respectively). On PQCT analysis, cortical bone

Fig. 2. Bone mass and bone density measurements in nonpregnant and pregnant guinea-pigs. Top: BMD of the whole left femur, measured by *DXA*. Middle: trabecular and cortical bone mineral density, assessed by *PQCT* at the distal femur. Bottom: trabecular bone volume (BV/TV), measured by histomorphometry at the secondary spongiosa of the proximal

tibia. Groups and number of data in each group, as in Figure 1. Statistical analysis: **a**, significant difference with the respective (nonpregnant or pregnant) +D group ($P < 0.05$, **aa**: $P < 0.01$); **b**, significant difference with the respective -D group ($P < 0.01$); **c**, significant difference with the nonpregnant -D group ($P < 0.01$).

density was lower in pregnant -D than in pregnant +D or nonpregnant -D animals, but this was not reversed by 1,25(OH)₂D₃ treatment or Ca/P supplementation. Trabecular bone density was not affected by the D-deficient diet, but Ca/P supplementation increased trabecular bone density. Histomorphometry at the secondary spongiosa confirmed that trabecular bone volume was increased in guinea pigs in the -D+Ca/P group.

The growth plate width at the proximal tibia was higher in pregnant -D than in pregnant +D or nonpregnant -D guinea pigs, and was partly reversed by 1,25(OH)₂D₃ treatment and Ca/P supplementation (Figs. 3, 4). Osteoid surface was lower in pregnant than in nonpregnant +D guinea pigs, confirming our previous observation [24]. -D animals had increased osteoid thickness and surface compared with +D

Fig. 3. Other histomorphometry data at the proximal tibia in nonpregnant and pregnant guinea pigs: GPW, growth plate width; OS/BS, osteoid surface; O.Th., osteoid thickness. Groups and number of data in each group, as in Figure 1. Statistical analysis: **a**, significant difference with the respective

(nonpregnant or pregnant) +D group (**a**: $P < 0.05$, **aa**: $P < 0.01$); **b**, significant difference with the respective (nonpregnant or pregnant) -D group (**b**: $P < 0.05$, **bb**: $P < 0.01$); **c**, significant difference with the respective (-D or +D) nonpregnant group ($P < 0.01$).

animals, the difference being more robust in pregnant animals. $1,25(\text{OH})_2\text{D}_3$ treatment partly reversed the hyperosteoidosis of -D animals, but Ca/P supplementation in -D animals failed to show a significant effect on osteoid surface and thickness. There was no difference among groups in the trabecular surface covered by TRAP-positive cells (ANOVA: $P = 0.24$; data not shown). Dynamic morphometry at the secondary spongiosa confirmed that bone mineralization was markedly lower in pregnant animals of all groups (Fig. 5), again in line with our previous observation [24]. The -D diet did not affect mineral formation rate in nonpregnant guinea pigs, but accentuated the pregnancy-induced decline in mineral formation rate, so that mineral formation rate was lower in pregnant than in nonpregnant -D guinea pigs. This was the result of a lower mineralizing surface, whereas the mineral apposition rate was not affected by the D-deficient diet. Ca/P supplementation in -D animals suppressed the mineral formation rate compared with +D animals; in pregnant animals, the mineral formation rate of -D and -D+Ca/P guinea pigs was comparable.

Fetal Guinea Pigs

Litter size was 3.8 ± 0.3 in the +D, -D, and -D+Ca/P groups and 5.0 ± 0.6 in the -D+ $1,25$ group (ANOVA: $P < 0.001$, with a significant difference between the -D+ $1,25$ and both the +D and -D groups). Fetuses of -D guinea pigs weighed less than +D fetuses, which was reversed by Ca/P supplementation, but not by $1,25(\text{OH})_2\text{D}_3$ treatment (Table 1). Placental weight was not significantly different among the groups (means between 3.9 and 4.1 g; ANOVA: $P = 0.73$).

Fetuses had higher levels of Ca^{2+} and P than their mothers, and maternal and fetal levels of Ca^{2+} ($r = 0.50$, $P = 0.03$, $n = 33$) and phosphate ($r = 0.72$, $P < 0.001$) were correlated. -D fetuses had higher Ca^{2+} but lower P levels than +D fetuses. Both $1,25(\text{OH})_2\text{D}_3$ treatment and Ca/P supplementation in pregnant guinea pigs normalized fetal Ca^{2+} and P levels. Fetal osteocalcin levels were 8-fold higher than in their mothers; there was no significant correlation between maternal and fetal levels. Osteocalcin concentrations were lower in -D than in +D fetuses, and were restored to +D levels in -D+ $1,25$ and -D+Ca/P fetuses. There was no significant differ-

Fig. 4. Representative sections of the proximal tibia of a 57-day-old pregnant +D (**top**) and -D (**bottom**) guinea pig, demonstrating the wide growth plates and the marked increase in osteoid tissue. Modified Goldner trichrome, magnification $\times 65$.

ence in amniotic fluid deoxypyridinoline concentrations among groups (ANOVA: $P = 0.26$; data not shown). Figure 1 shows that fetal $25(\text{OH})_2\text{D}_3$, DBP and $1,25(\text{OH})_2\text{D}_3$ levels were lower than the respective maternal values, but the calculated free $1,25(\text{OH})_2\text{D}_3$ index was higher (data not shown). All -D fetuses had undetectable levels of $25(\text{OH})_2\text{D}_3$, and 8/9 had undetectable levels of $1,25(\text{OH})_2\text{D}_3$. $1,25(\text{OH})_2\text{D}_3$ treatment normalized $1,25(\text{OH})_2\text{D}_3$ levels. Maternal and fetal concentrations of the vitamin D metabolites were highly correlated ($r = 0.85$ for both $25(\text{OH})_2\text{D}_3$ and free $1,25(\text{OH})_2\text{D}_3$ index and $r = 0.65$ for $1,25(\text{OH})_2\text{D}_3$, all $P < 0.001$, $n = 33$).

Whole-body BMC was reduced in -D fetuses compared with +D fetuses, which was restored to +D values by $1,25(\text{OH})_2\text{D}_3$ treatment and Ca/P supplementation (Fig. 6). Because body weight was also lower in -D fetuses, the BMC/weight ratio was comparable in -D and +D fetuses. Since -D + $1,25$ fetuses had normal BMC but reduced body weight, the BMC/weight ratio was higher than in +D and -D fetuses. Calculated fetal BMD values were comparable to BMC values (data not shown). Histomorphometry of the proximal tibia showed that there was an expansion of the hypertrophic chondrocyte area in -D fetuses, which was normalized by both

Fig. 5. Mineral apposition rate (MAR) and mineral formation rate (MFR/BS) at the proximal tibial metaphysis in nonpregnant and pregnant guinea pigs. Groups and number of data in each group, as in Figure 1. Statistical analysis: **a**, significant difference with the respective (nonpregnant or pregnant) +D group (**a**: $P < 0.05$, **aa**: $P < 0.01$); **b**, significant difference with the respective -D group (**b**: $P < 0.05$, **bb**: $P < 0.01$); **c**, significant difference between pregnant vs. nonpregnant animals in the same diet group (**c**: $P < 0.05$, **cc**: $P < 0.01$).

1,25(OH)₂D₃ treatment and Ca/P supplementation (Figs. 7 and 8). There was also a clear increase in the osteoid surface and thickness in -D fetuses, and this was partly reversed by 1,25(OH)₂D₃ treatment and Ca/P supplementation.

Discussion

Guinea pigs fed the -D diet for 7–8 weeks had very low or undetectable 25(OH)D₃ and significantly reduced 1,25(OH)₂D₃ levels. However, they were not hypocalcemic, presumably because of secondary hyperparathyroidism. We made three novel observations regarding the effects of a -D diet in pregnant guinea pigs. The first was that the depletion in vitamin D metabolites engendered by the -D diet is more robust in pregnant animals: 25(OH)D₃ levels were undetectable in all pregnant but in only 2/7 nonpregnant guinea pigs, and the well-characterized rise in 1,25(OH)₂D₃ levels during pregnancy [36, 37] was absent in -D animals. In

Fig. 6. Fetal whole-body BMC, assessed by DXA, and BMC per body weight (after evisceration *post mortem*). Groups as in Figure 1, *n* is the number of data in each group. Statistical analysis: **a**, significant difference with +D fetuses ($P < 0.01$); **b**, significant difference with -D fetuses (**b**: $P < 0.05$, **bb**: $P < 0.01$).

addition, pregnant but not nonpregnant guinea pigs were hypophosphatemic.

The second observation was that the skeletal effects of the D-deficient diet are more dramatic during concurrent pregnancy. In nonpregnant guinea pigs, the only change was a modest rise in osteoid surface and thickness (osteomalacia). In counterpart, in pregnant animals, the -D diet induced (1) an increase in growth plate width (rickets), (2) a more robust increase in osteoid surface/thickness, (3) a dampening of the mineral formation rate, superimposed on the effect of pregnancy [24], and (4) a reduction in femoral BMD on DXA and in cortical bone density on PQCT. The pregnancy-induced skeletal effects are not surprising, given the substantial fetal Ca/P needs in guinea pigs. Indeed, the Ca content of an average guinea pig litter at day 60 is 6 g [38], though we measured that femora of the nonpregnant guinea pigs in this series contain but 245 ± 7 mg Ca ($n = 6$). By contrast, in rats, a -D diet for 3 weeks—resulting in very low or undetectable 25(OH)D₃ but unaltered 1,25(OH)₂D₃ levels—did not affect pregnant animals differently than nonpregnant animals, although the endosteal bone formation rate was reduced only in pregnant rats [39].

Fig. 7. Histomorphometry of the tibia in fetal guinea pigs: hypertrophic chondrocyte area (% of total chondrocyte area) at the proximal tibial growth plate; OS/BS, osteoid surface; O.Th., osteoid thickness. Groups as in Figure 1, *n* is the number of data in each group. Statistical analysis: **a**, significant difference with +D fetuses (**a**: $P < 0.05$, **aa**: $P < 0.01$); **b**, significant difference with -D fetuses (**b**: $P < 0.05$, **bb**: $P < 0.01$).

These findings confirm some clinical observations. Fogelman et al. in Israel noted that D deficiency was clinically more apparent during pregnancy and breastfeeding [3]. Gannagé-Yared et al. found that high parity was a powerful predictor of low 25(OH)D₃ levels in a cohort of 217 Lebanese women aged 30–50 years [5]. D deficiency was also common in Saudi-Arabian women in the reproductive period, and the BMD at both lumbar spine and proximal femur correlated inversely with parity [40].

The third observation was that fetuses of -D guinea pigs were growth-retarded, with a commensurate delay in bone mineralization. Indeed, plasma osteocalcin levels were reduced, and both weight and BMC were 10–15% lower, but BMC/weight was normal. These fetuses were unequivocally D-deficient: they had undetectable 25(OH)D₃ and very low or undetectable 1,25(OH)₂D₃ levels. In addition, they were hypercalcemic and hypophosphatemic, indicative of secondary hyperparathyroidism. Hyperparathyroidism has also been observed in newborns of D-deficient Asian mothers [41]. Unfortunately, we were unable to actually measure PTH levels in these guinea pigs. Findings on bone histomorphometry

mirrored those of their mothers: fetuses of D-deficient mothers had rickets (expansion of the hypertrophic cartilage zone) and osteomalacia (increased osteoid surface/thickness).

Subsequently, we infused nonpregnant, pregnant, and fetal -D guinea pigs with 1,25(OH)₂D₃, which corrected circulating 1,25(OH)₂D₃ to physiological or slightly supraphysiological levels. Although the same dose was given, we found that pregnant animals had higher plasma 1,25(OH)₂D₃ levels than nonpregnant animals. This suggests that the endogenous 1 α -hydroxylase activity was stimulated during pregnancy, as in D-replete animals. The observation that fetal 1,25(OH)₂D₃ levels were also normalized, corroborates the paradigm that circulating 1,25(OH)₂D₃ in fetuses is determined by transplacental transport rather than by synthesis in the fetal kidney. Indeed, this study and our previous studies in humans, guinea pigs, and rats demonstrate highly significant correlations between maternal and fetal circulating 1,25(OH)₂D₃ levels [23–25]. Because the treatment was given for the last 4 weeks of pregnancy only, the maternal skeletal effects of D deficiency were partly normalized, with a re-

Fig. 8. Representative sections of the proximal tibia of a +D (top) and -D (bottom) guinea pig fetus demonstrating the expansion of the hypertrophic chondrocyte area and the increase in osteoid tissue. The dashed line demarcates the zone of hypertrophic chondrocytes from the zone of maturation. Modified Goldner trichrome, magnification $\times 65$.

duction in growth plate width and in osteoid surface/thickness. But the treatment was primarily intended to study its effect on fetal growth and mineralization, since fetal mineralization starts at about day 26 [26]. We found that $1,25(\text{OH})_2\text{D}_3$ infusion normalized fetal whole-body BMC, plasma osteocalcin levels, and the expansion of the hypertrophic cartilage zone, and partly normalized osteoid surface/thickness. Fetal growth was unaffected by $1,25(\text{OH})_2\text{D}_3$ treatment, but litter size was higher in this group; it is known that perinatal weight gain is inversely related to litter size in small animals [14]. Larger litters in this group may also be the explanation for the lower Ca^{2+} levels in

their mothers, compared with D-replete and D-deficient mothers. Indeed, maternal Ca^{2+} concentrations are negatively affected by litter size in guinea pigs on a normal [42] or low Ca/P diet [24].

In a final experiment, we supplemented the -D diet with lactose and extra Ca and P. This diet raised plasma P levels and slowed down bone mineralization in trabecular bone. Consequently, trabecular bone volume on histomorphometry and trabecular bone density on PQCT were higher than in D-deficient and D-replete animals. Compared with pregnant animals on the non-supplemented -D diet, there was a normalization of the femoral BMD on DXA, and a trend toward normal-

ization of the growth plate width and osteoid surface/thickness on morphometry and the cortical bone density on PQCT. Presumably, an even higher Ca/P intake is required to completely reverse the effects of D deficiency during pregnancy. In their fetuses, the diet fully normalized growth, and several parameters of mineralization, including whole body BMC, plasma osteocalcin levels, and hypertrophic chondrocyte area. The data indicate that increasing the mineral supply to the mother largely reverses the effects of D deficiency on fetal growth and mineralization. Alternatively, the data strongly suggest that endogenous vitamin D metabolites *per se* are not necessary for fetal growth and mineralization in the guinea pig, and infer that the transplacental transport of Ca and P is largely—if not completely—vitamin D-independent. However, fetal osteoid surface/thickness was only partly normalized. It cannot, therefore, be excluded that vitamin D metabolites do modulate osteoblast function during fetal life: indeed, 1α -hydroxylase gene and VDR expression have been demonstrated in fetal mouse and rat long bones, respectively [43, 44]. Notwithstanding this possibility, the current study reaffirms previous evidence that the primary effect of $1,25(\text{OH})_2\text{D}_3$ during pregnancy consists of stimulating maternal intestinal Ca and P absorption, thus providing the fetus(es) with mineral supplies for bone mineralization in a hypercalcemic and hyperphosphatemic environment, while preventing significant bone demineralization as well as hypocalcemia and hypophosphatemia in the mother.

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