

Effect of 25-hydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₃ maternal loads on maternal and fetal vitamin D metabolite levels in the rat.

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Summary. Two groups of female rats were used to investigate vitamin D metabolism in the pregnant animals and in their fetuses. In the first group, 3 µg of 25-hydroxyvitamin D₃ (25-OH-D₃) per kg of body weight were injected into intact or nephrectomized (NX) pregnant rats 3 h before sacrifice on day 21 of pregnancy; in the second group, 2 and 6 ng, respectively, of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) per day were infused continuously into pregnant rats between days 17 and 21 of pregnancy. The findings in the fetuses were obtained by quantitative analysis of extracts (Extrelut) of total fetal body lipids; the extracts were purified on Sep Pak and vitamin D sterols were further separated by high-pressure liquid chromatography. Three hours after the dams were injected with 25-OH-D₃, the maternal plasma concentration (mean ± SD) of 1,25(OH)₂D₃ was 221 ± 84 pg/ml. In NX pregnant rats, the 1,25(OH)₂D₃ levels were still elevated: 95.6 ± 49.0 pg/ml vs 45 ± 22 pg/ml in control rats. In fetuses from intact or NX dams, the levels of 25-OH-D₃ and 1,25(OH)₂D₃ were not different from the results obtained in the control fetuses but 24,25(OH)₂D₃ concentrations were increased (6.7 ± 1.2 ng vs 2.2 ± 0.7 ng/g body weight). After maternal infusion of 2 or 6 ng/day of 1,25(OH)₂D₃ (n = 8), plasma concentrations (mean ± SD) of the metabolite were 64 ± 31 and 517 ± 356 pg/ml, respectively, the second being significantly higher than that of the control rats; 25-OH-D₃ and 24,25(OH)₂D₃ levels did not change. 1,25(OH)₂D₃ contents (mean ± SD) in fetuses from the treated dams were not different from those of control fetuses (10 ± 2 pg/g body weight).

Our results suggest that pregnant rats and their fetuses were protected against an excessive increase of 1,25(OH)₂D₃ concentrations in the maternal plasma; although there was some individual hypercalcemia, no significant increase in mean calcemia was detected in the dams, and 1,25(OH)₂D₃ either did not cross the placental barrier or was rapidly metabolized because we did not find any changes in the fetal content. As in intact or NX pregnant rats, 25-OH-D₃ was metabolized into 1,25(OH)₂D₃, the increase of 24,25(OH)₂D₃ in the fetuses might be associated with a protective mechanism.

Introduction.

Recently, plasma levels of 1,25-dihydroxycholecalciferol (1,25(OH)₂D₃) have been reported to be significantly elevated in rats (Pike *et al.*, 1979) and in humans (Kumar *et al.*, 1979) during pregnancy. Weisman *et al.* (1978) and Gray, Lester

and Lorenc (1979) working independently demonstrated that nephrectomy of pregnant, vitamin D-deficient rats reduced but did not abolish the *in vivo* conversion of (³H)-25-hydroxycholecalciferol into (³H)-1,25(OH)₂D₃. They concluded that the fetoplacental unit was the most likely site of 1,25(OH)₂D₃ production in anephric animals.

Although 1,25(OH)₂D₃ increases in the maternal circulation, only very small amounts of the metabolite are reported in the fetus. In contrast, 24,25-dihydroxycholecalciferol (24,25(OH)₂D₃) accumulates in the fetal tissues, particularly in the skeleton (Weisman *et al.*, 1976 ; Noff and Edelstein, 1978). It was suggested that fetuses might have a mechanism protecting them against vitamin D intoxication and show a pattern of selectivity in the placental transport of cholecalciferol and its hydroxylated metabolites.

Little is known about the fetal metabolism of vitamin D derivatives. In order to determine the fetal metabolism of vitamin D administered to pregnant rats, we investigated the effects of acute administration of 25-OH-D₃ to intact or nephrectomized (NX) pregnant rats and of continuous injection of 1,25(OH)₂D₃. Three major metabolites (1,25-(OH)₂D₃ ; 24,25(OH)₂D₃ and 25-OH-D₃) were measured in the whole fetal body along with other biological parameters (calcemia, phosphoremia and phosphatase activity in fetal plasma and bones) which might be affected by the above treatments.

Material and methods.

Animals and surgical procedure.

Two treatments were carried out on Wistar rats : (1) intact or recently nephrectomized pregnant rats were injected with 25-OH-D₃ (3 µg/kg body weight) or with the same volume of ethanol 3 h before sacrifice on day 21 of pregnancy. The controls were non-pregnant rats of the same age and treated according to the same protocol ; (2) female rats were chronically infused from day 17 of pregnancy with 1,25(OH)₂D₃ (2 and 6 ng/rat/day, respectively) dissolved in propylene glycol. An Alzet osmotic minipump was used for infusion. The sham-operated control rats received no pump.

The animals were fed a commercial vitamin D-replete diet containing 1.5 % of Ca and 0.9 % of P. All the rats were sacrificed on day 21 of pregnancy after the fetal and maternal blood was collected.

Vitamin D metabolites were measured in both the maternal plasma and the fetal extracts :

— *Procedure for maternal plasma* : two ml of plasma from each rat were thawed at room temperature, and trace amounts (2 000-3 000 cpm) of radiolabelled 25-(³H)-hydroxyvitamin D₃ (25-OH-D₃), 24R,25 (³H)-dihydroxyvitamin D₃ (24,25(OH)₂D₃) and 1α,25 (³H)-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) were added to monitor procedural loss. Lipid-soluble vitamin D metabolites were extracted with 1 volume of ethanol : hexane : ether (1 : 2.5 : 2.5) in the presence of 1/5 volume of NH₄OH. The lipid extract was taken to dryness under nitrogen.

— *Procedure for the extraction of fetal metabolites* : the fetuses were removed rapidly and the fetal blood was collected into an heparinized Pasteur pipette after section of the brachial vessels. One or two fetuses from each dam were rinsed with a saline solution to avoid contamination from maternal blood and then were chopped, homogenized and sonicated in 5 ml of Tris buffer, pH 7, at 0°C (KCl 0.3M, EDTA 0.0015M, dithiothreitol 0.0005M, Tris 0.1M). Radiolabelled 25-OH-D₃, 24,25(OH)₂D₃ and 1,25(OH)₂D₃ (Amersham) were added to these homogenates, and the samples were allowed to equilibrate for 45 min at 4°C. Each extract, completed to 16.8 ml by the addition of Tris buffer with 50 g/l of bovine serum albumin, was deposited on a ready-to-use, Extrelut 20 column (Merck) for lipid extraction. The solvent system was : ether/hexane/ethanol (22.5 ml/22.5 ml/6 ml). The fraction containing vitamin D metabolites was then evaporated under reduced pressure. Extraction recovery (mean ± SD) was 49 ± 5 % and 84 ± 4 % for 24,25(OH)₂D₃ and 1,25(OH)₂D₃, respectively.

— *Separation of the metabolites* : the vitamin D metabolites in the lipid fraction of the plasma or of the whole fetus extraction were separated on Sep-Pak according to Adams *et al.* (1981). The fractions containing 24,25(OH)₂D₃ and 1 α ,25(OH)₂D₃ were purified further by high-pressure liquid chromatography (HPLC) using a μ -porasil column in 10 % isopropanol in a heptane isocratic solvent system. Overall recovery of 25-OH-D₃, 24,25(OH)₂D₃ and 1,25(OH)₂D₃ was 91 ± 5, 63 ± 14 and 85 ± 10 %, respectively, for the maternal plasma and 59 ± 11, 40 ± 12 and 62 ± 6 %, respectively, for the tissue extract. 25-OH-D₃ and 24,25(OH)₂D₃ were measured by a competitive protein binding assay using normal rat kidney cytosol as the receptor source ; standard sensitivity ranged between 3.2 and 0.025 ng/assay tube. 1,25(OH)₂D₃ was measured by a radioreceptor assay using normal chick intestinal cytosol as the receptor source (Shepard and Deluca, 1980) ; assay sensitivity ranged between 200 and 1.56 pg/assay tube. Non-specific binding was determined for each sample by adding an excess of the cold metabolite.

Biochemical determination. — Plasma calcium and phosphate concentrations were measured by colorimetric methods (Chen *et al.*, 1956). Alkaline phosphatase activity was determined according to Jouzier *et al.* (1968) in a bone extract obtained by homogenization and sonication in a 0.1 % solution of sucrose 0.25M and Triton X 100.

Results.

25-OH-D₃ Administration. — Vitamin D metabolite concentrations in the maternal plasma 3 h after 25-OH-D₃ administration (3 μ g/kg body weight) in intact or NX pregnant rats are shown in table 1. The results have been compared with those obtained in non-pregnant rats. The mean 1,25(OH)₂D₃ concentration (mean ± SD) in non-injected, intact pregnant rats was 45 ± 22 pg/ml. On day 21 of pregnancy, the values of nephrectomized pregnant rats did not differ. After injection of 25-OH-D₃, the plasma levels of 1,25(OH)₂D₃ were 221 ± 84 and 95.6 ± 49.0 pg/ml in intact and NX rats, respectively (R = 2.3). The ratio of

TABLE 1

Effect of 25-OH-D₃ administration in intact or NX rats (pregnant or not) on vitamin D metabolites and plasma calcium concentrations.

Female status	25-OH-D ng/ml	24,25(OH) ₂ D ng/ml	1,25(OH) ₂ D pg/ml	Ca mg/l
Pregnant (control)	6.05 ± 1.16 (6)	2.29 ± 1.10 (6)	45 ± 22 (7)	97.5 ± 4.5 (6)
Pregnant and NX	2.87 ± 1.15 ^c (6)	1.71 ± 1.00 (6)	46 ± 6 (5)	100.3 ± 3.4 (6)
Pregnant and 25-OH-D ₃ injected	3.89 ± 1.88 ^a (3)	1.82 ± 0.94 (4)	221 ± 84 ^c (4)	99.1 ± 2.9 (8)
Pregnant, 25-OH-D ₃ injected and NX	4.60 ± 0.93 (5)	1.19 ± 0.34 (4)	96 ± 49 ^{bβx} (6)	105.1 ± 5.8 (10)
Non-pregnant and 25-OH-D ₃ injected	11.08 ± 1.16 (4)	3.74 ± 0.69 (4)	67 ± 36 (4)	103.7 ± 6.8 (4)
Non-pregnant, 25-OH-D ₃ injected and NX	9.08 ± 2.39 (4)	3.53 ± 0.40 (4)	13 ± 5 ^α (4)	113.1 ± 3.4 (4)

Results are mean ± SD ; the number in parenthesis is the number of experiments :

^a : p < 0.05 compared with control pregnant rats ; ^b : p < 0.01 compared with control pregnant rats ; ^c : p < 0.001 compared with control pregnant rats ; ^β : p < 0.02 NX compared with intact pregnant rats ; ^α : p < 0.02 NX compared with intact non-pregnant rats ; ^x : p < 0.01 compared with non-pregnant rats.

(Student's t-test).

plasma 1,25(OH)₂D₃ concentrations between intact and non-pregnant rats was 5 in similar conditions. The concentrations of 25-OH-D₃ were decreased after nephrectomy (2.87 ± 1.15 vs 6.05 ± 1.6 ng/ml in the controls). No significant changes were noted in the 1,25(OH)₂D₃ levels of whole fetus body after 25-OH-D₃ administration in either intact or NX rats (10.8 ± 5.6 pg/g body weight vs 7.8 ± 5.0 pg/g body weight ; n = 4) as compared with non-injected dams (8.7 ± 5.0 pg/g body weight ; n = 8), but the level of 24,25(OH)₂D₃ was increased after 25-OH-D₃ administration in intact and NX rats as compared with non-injected dams (table 2). Neither calcemia nor phosphoremia in the dams and fetuses was affected by maternal injection or nephrectomy.

TABLE 2

Effect of 25-OH-D₃ injection in intact or NX pregnant rats on vitamin D metabolite concentrations in the whole fetal body and on plasma calcium and phosphorus concentrations 3 h after administration and surgery.

Dam status	25-OH-D ng/g	24,25(OH) ₂ D ng/g	1,25(OH) ₂ D pg/g	Ca mg/l	P mg/l
Control	9.5 ± 2.1 (8)	2.2 ± 0.7 (6)	8.7 ± 5.0 (8)	103.80 ± 9.0 (19)	92.3 ± 9.5 (8)
Injected and intact	5.2 ± 0.5 ^a (4)	6.8 ± 2.4 ^b (4)	10.8 ± 5.6 (4)	107.3 ± 9.6 (12)	103.6 ± 13.3 (11)
Injected and NX	4.9 ± 0.7 (4)	6.7 ± 1.2 (4)	7.8 ± 0.5 (4)	111.1 ± 11.5 (9)	100.5 ± 15.7 (8)

Student's t-test : ^a : p < 0.01 ; ^b : p < 0.001, compared with control.

Chronic administration of 1,25(OH)₂D₃. — Plasma concentrations of vitamin D metabolites in pregnant rats after chronic administration of 1,25(OH)₂D₃ are shown in table 3. The plasma levels (mean \pm SD) of 1,25(OH)₂D₃ in the dams were significantly elevated after 6 ng/day (517 \pm 356 pg/ml) as compared with the group given 2 ng/day (64 \pm 31 pg/ml) and the control group (45 \pm 22 pg/ml). The levels of 25-OH-D₃ or 24,25(OH)₂D₃ did not change. The mean levels of calcemia were within the normal limits (103.60 \pm 8.50 mg/l) because of the wide range of individual values; however, two rats were clearly hypercalcemic (114.2, 109.2 mg/l) with the higher dose.

Fetal plasma concentrations of calcium, phosphate and alkaline phosphatase activity are shown in table 4 along with acid and alkaline phosphatase activities in fetal bone extracts. There was no difference between fetuses from treated and non-treated dams, except for the alkaline phosphatase activity in bone

TABLE 3

Effect of chronic 1,25(OH)₂D₃ administration (2 and 6 ng/day) in pregnant rats on vitamin D metabolites and maternal plasma calcium concentrations.

	25-OH-D ng/ml	24,25(OH) ₂ D ng/ml	1,25(OH) ₂ D pg/ml	Ca mg/l
Control 1,25(OH) ₂ D injection	6.05 \pm 1.16 (16)	2.29 \pm 1.10 (6)	45 \pm 22 (7)	97.5 \pm 4.5 (6)
2 ng/d	6.0 \pm 0.58 (4)	3.00 \pm 0.24 (4)	64 \pm 31 (4)	93.0 \pm 1.2 (5)
6 ng/d	6.3 \pm 1.8 (6)	2.8 \pm 1 (6)	517 \pm 356 ^c (6)	103.6 \pm 8.5 NS (4)

Results are mean \pm SD.

^c : p < 0.001 compared with control pregnant rats.

TABLE 4

Effect of chronic administration of 1,25(OH)₂D₃ in pregnant rats from day 17 of pregnancy on biological parameters in fetuses at day 21 of gestation.

	Calcemia mg/l	Phosphatemia mg/l	Alkaline phosphatase in plasma UI/l	Phosphatases in bone	
				Alkaline (UI/bone)	Acid (UI/bone)
Control 1,25(OH) ₂ D ₃	103.8 \pm 9 (18)	92.4 \pm 9.5 (18)	245 \pm 68 (10)	3.71 \pm 0.44 (8)	0.25 \pm 0.024 (8)
2 ng/day	107.3 \pm 5.0 (7)	104.6 \pm 7.3 (7)	254 \pm 32 (10)	3.42 \pm 0.65 (7)	0.20 \pm 0.04 (7)
6 ng/day	102.2 \pm 9.4 (12)	92.2 \pm 13.0 (12)	239.1 \pm 49 (12)	4.39 \pm 0.83* (10)	0.28 \pm 0.09 (10)

Results are mean \pm SD.

* p < 0.05 in comparison with control rat.

Phosphatase activities in bone were measured in a femora extractum in a sucrose 0.25M triton \times 100 0.1 % solution.

homogenates which was significantly elevated after the dams were treated with 6 ng/day of $1,25(\text{OH})_2\text{D}_3$.

The concentrations of the vitamin D metabolite in whole fetuses were not affected by the $1,25(\text{OH})_2\text{D}_3$ treatment, even at the highest dose. The concentrations were 10.0 ± 4.0 pg/g body weight in fetuses of treated dams vs 8.7 ± 5 pg/g body weight in control fetuses.

Discussion.

The results of this study show that 1α -hydroxylase activity in the kidney and/or the placenta is effective at the end of pregnancy. When an injection of 25-OH- D_3 was given just after nephrectomy, the levels of $1,25(\text{OH})_2\text{D}_3$ were reduced by half in pregnant rats, whereas in non-pregnant animals they decreased 5-fold. Plasma $1,25(\text{OH})_2\text{D}_3$ in non-pregnant rats may be related to the fact that the product was not entirely metabolized within 2 h after nephrectomy. These results are consistent with those reported by other investigators concerning the increased formation of $1,25(\text{OH})_2\text{D}_3$ during pregnancy, but the stimulatory factor was not known. On the other hand, we confirm the existence of an extra-renal 1α -hydroxylase which was demonstrated earlier *in vivo* (Gray *et al.*, 1979, 1981 ; Weisman *et al.*, 1978) in vitamin D-deficient pregnant rats and *in vitro* in the human decidua and placenta (Weisman *et al.*, 1979). Maternal nephrectomy reduced but did not abolish the conversion of 25-OH- D_3 to its dihydroxylated derivatives, as previously demonstrated.

25-OH- D_3 concentrations were reduced in both the maternal plasma and the fetal body after 25-OH- D_3 was administered to the dams ; the metabolite seemed to be preferentially 1α -hydroxylated in pregnant rats by either the maternal kidney and/or the placenta. The 25-OH- D_3 levels in the fetuses reflect those in the maternal plasma. Haddad *et al.* (1971) showed that 1 h after 25-OH- D_3 administration to intact vitamin D-repleted rats, their fetuses only contained 2 % of the dose administered in the form of ^3H -25-OH- D_3 but that more polar metabolites were already present.

In NX rats, 25-OH- D_3 was metabolized in the placenta and did not seem to enter the fetal circulation since no increase in fetal body levels of $1,25(\text{OH})_2\text{D}_3$ was shown. Nevertheless, it could have been metabolized by the fetal kidneys or extra-renal tissues and we would not have been able to detect any changes in the fetal contents. Whether $1,25(\text{OH})_2\text{D}_3$ of placental origin directly affects the placental cells is unknown at present.

Kubota *et al.* (1982) tried giving large amounts (650 nmol daily) of vitamin D to pregnant rabbits : plasma concentrations of $1,25(\text{OH})_2\text{D}_3$ were not affected in either the dams or the fetuses, but both maternal and the fetal plasma levels of 25-OH- D_3 and $24,25(\text{OH})_2\text{D}_3$ were increased. Our results differ from the above results of Kubota *et al.* (1982) since a preferential increase of $1,25(\text{OH})_2\text{D}_3$ in the maternal plasma was shown after the dams were injected with 25-OH- D_3 at day 21 of pregnancy ; Kubota *et al.* demonstrated an inhibition of 1α -hydroxylase in rabbit kidneys after vitamin D treatment. On the other hand, we obtained an increase in $24,25(\text{OH})_2\text{D}_3$ concentration in fetal bodies from intact or NX rats,

indicating an active 24-hydroxylase either in the placenta or the fetal kidney ; fetal hypercalcemia enhanced the *in vivo* synthesis of 24,25(OH)₂D₃ and perhaps that of 1,24,25(OH)₃D₃, although fetal rabbit kidney *in vitro* preferentially transforms 25-OH-D₃ to its 1 α -hydroxylated derivative (Sunaga *et al.*, 1979).

The dams and fetuses in the present study seemed to be protected against excessive amounts of 1,25(OH)₂D₃ in the maternal plasma ; even a concentration of 517 \pm 356 pg/ml did not lead to a significant increase in the mean levels of calcemia in the dams, but some rats were clearly hypercalcemic ; the only change found in the fetuses was an increase in bone alkaline phosphatase which might be correlated with an increase in osteoblast activity or number. These results could be compared to the report of Salle *et al.* (1981) concerning a woman suffering from idiopathic hypoparathyroidism who was treated with calcitriol during pregnancy ; her infant boy showed an increase in long bone and skull densities which was interpreted as the result of excessive mineralization. It is possible that placental transfer occurred due to an enlarged hormone pool. In animal experiments, similar observations have been reported ; the chronic administration of 1,25(OH)₂D₃ led to an increase of fetal bone resorption (Rebut-Bonneton *et al.*, 1983) which was supposed to be caused by the transplacental passage of either 1,25(OH)₂D₃ or another metabolite. The effect of 1,25(OH)₂D₃ on alkaline phosphatase activity in fetal bone is controversial since it has been shown that 1,25(OH)₂D₃ administration in pregnant rats increases fetal bone resorption (Rebut-Bonneton *et al.*, 1983) and the number of active resorptive cells (Rebut-Bonneton *et al.*, 1984). An increase in acid phosphatase activity in fetal bone might have occurred. However, the direct effects on bone alkaline phosphatase activity are conflicting and suggest that vitamin D₃ metabolites have dual stimulatory and inhibitory effects which may be related with the mode of administration (acute or continuous) or with the dose of vitamin D₃ used.

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Résumé. *Apport de 25 hydroxyvitamine D₃ et 1,25 dihydroxyvitamine D₃ chez la rate gestante : effets sur les taux des métabolites de la vitamine D chez la mère et le fœtus.*

Deux expériences ont été réalisées pour étudier le métabolisme de la vitamine D chez la rate gestante (mère et fœtus). Une injection de 25-hydroxyvitamine D₃ (25-OH-D₃-3 μ g/kg de poids) chez la rate gestante intacte ou néphrectomisée (NX) — et, une infusion chronique de 1,25 dihydroxyvitamine D₃ (1,25(OH)₂D₃) (2 et 6 ng par jour) du 17^e au 21^e jour de gestation. Les résultats chez le fœtus ont été obtenus par dosages des métabolites de la vitamine D dans un extrait lipidique (extrelut) du corps entier du fœtus ; l'extrait était purifié sur Sep Pak puis les métabolites étaient séparés par chromatographie liquide haute pression. Trois heures après injection du 25-OH-D₃ à la rate, la concentration plasmatique en 1,25(OH)₂D₃ de la mère était 221 \pm 84 pg/ml. Chez la rate préalablement néphrectomisée, le taux de 1,25(OH)₂D₃ demeure élevé (95,6 \pm 49 pg/ml) en comparaison avec la rate témoin (45 \pm 22 pg/ml). Les concentrations de 25-OH-D₃ et 1,25(OH)₂D₃ chez le fœtus dont la mère a reçu le 25-OH-D₃ ne sont pas différentes de celles des fœtus témoins mais, le taux de 24,25(OH)₂D₃ est augmenté (6,7 \pm 1,2 ng/g au lieu de 2,2 \pm 0,7 pour les témoins).

Après injection chronique de 1,25(OH)₂D₃ les concentrations plasmatiques en 1,25(OH)₂D₃ sont 64 \pm 31 et 517 \pm 356 pg/ml (moyenne \pm SD) respectivement après

administration de 2 et 6 ng par jour ; soit une différence significative en comparaison avec le témoin (45 ± 22 pg/ml) pour la plus forte dose. Les concentrations chez le fœtus ne sont pas différentes entre les groupes.

A l'examen de nos résultats il semblerait que rate gestante et fœtus seraient protégés contre des concentrations excessives en $1,25(\text{OH})_2\text{D}_3$ dans le plasma maternel. Le $1,25(\text{OH})_2\text{D}_3$ ne traverserait pas la barrière placentaire ou, il serait rapidement métabolisé. Que la rate soit intacte ou NX, le 25-OH-D₃ est transformé en $1,25(\text{OH})_2\text{D}_3$ chez la mère ; la présence chez le fœtus d'une concentration augmentée de $24,25(\text{OH})_2\text{D}_3$ pourrait être associée à un mécanisme de défense.

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