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Tolerance to 1,25 dihydroxyvitamin D_3 glycosides from *Solanum glaucophyllum* by the growing pig

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ABSTRACT

Solanum glaucophyllum leaves contain high levels of glycosidically bound 1,25 dihydroxyvitamin D_3, the most important vitamin D metabolite. The tolerance to this source was evaluated during six weeks with fifty weaned pigs fed increasing levels (0, 2.5, 5, 10 and 20 μg 1,25$(OH)_2D_3$/kg diet). The diet contained, per kg, 9.7 g Ca, 3.5 g digestible P and 2000 IU cholecalciferol. Ten additional pigs were fed a diet containing 1000 IU cholecalciferol/kg, without 1,25$(OH)_2D_3$. Weekly plasma and final kidney, bone and urinary mineral contents, bone density and breaking strength served as indicators for possible adverse effects of the supplement. All animals grew well and remained clinically healthy. The measured parameters remained unchanged when 1000 replaced 2000 IU cholecalciferol/kg and when 1,25$(OH)_2D_3$ was fed up to 10 μg/kg. Twenty μg 1,25$(OH)_2D_3$ increased plasma Ca and decreased plasma P from the 2nd and the 4th experimental week onwards, respectively. Twenty μg 1,25$(OH)_2D_3$ increased final plasma Ca and 1,25$(OH)_2D_3$ and reduced final plasma P by respectively 19, 56 and 13%. Twenty μg 1,25$(OH)_2D_3$ also increased kidney Ca and urinary Ca by 43 and 69%, respectively, reduced bone breaking strength by 12% and tended to decrease bone ash by 3%. To conclude, 2000 IU D_3 was not beneficial compared to 1000 IU cholecalciferol; up to 10 μg 1,25$(OH)_2D_3$ per kg diet did not lead to observed adverse effects; 20 μg 1,25$(OH)_2D_3$ altered the homeostatic regulation of Ca and P thus, may lead to first signs of possible adverse effects, such as soft tissue calcification.

1. Introduction

Since the beginning of the 20th century, calcinosis was reported in grazing cattle from Argentina (Collier, 1927). Worker and Carrillo (1967) indicated that this disease is caused by the intake of the plant *Solanum glaucophyllum* (formerly *Solanum malacoxylon*), which contains glycosides of vitamin D_3 and its metabolites, mainly as 1,25 dihydroxyvitamin D_3 (Skliar et al., 1992). Glycosidase produced by rumen microbes and in the intestinal mucosa can cleave the sugar residue from the glycoside and thus release the steroidal fragment, such as 1,25 dihydroxyvitamin D_3 (Mello, 2003). Intoxication with calcinogenic plants, such as *Solanum glaucophyllum* result in joint erosions clinically manifested as stiffness, painful gait, kyphosis, anorexia and loss of body condition and calcification of arteries, heart, lungs, thymus and kidneys (Gimeno et al., 2000; Mello, 2003; Barros et al., 2006; Zanuzzi et al., 2008; Fontana et al., 2009; Zanuzzi et al., 2010; Zanuzzi et al., 2012). Vitamin D is however required for a proper Ca, P and Mg absorption and utilisation (NRC, National Research Council, 2012). Before becoming metabolically active, vitamin D_3 is first hydroxylated to 25 hydroxycholecalciferol (25$(OH)D_3$) in the liver. Then, in response to a decrease in serum Ca and the resulting increase in parathyroid hormone concentration, 25$(OH)D_3$ is further hydroxylated to 1,25 dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) in the kidney. The release of 1,25$(OH)_2D_3$ leads to an increased intestinal absorption of Ca, P and Mg. Vitamin D is commonly supplemented in livestock diets and commercial sources of vitamin D are available as cholecalciferol (D_3), as 25$(OH)D_3$ and as 1,25$(OH)_2D_3$. Cholecalciferol is the most common form of feed additive used in animal nutrition and the interest in using the metabolically active form relies on its potentially improved bioavailability. Sources of 1,25$(OH)_2D_3$ are of synthetic or plant origin. The leaves or the extracts of *Solanum glaucophyllum*, rich in the water soluble form of glycosides of 1,25$(OH)_2D_3$ were reported to be highly bioavailable in rats (Napoli et al., 1977; von Rosenberg et al., 2007) and in pigs (Fox and Care, 1979). A standardised powder of *Solanum glaucophyllum* is commercially available for livestock nutrition (Bachmann et al., 2013). Feeding this product may bypass relevant steps of the homeostatic regulations of Ca and may increase the risk of incidences of hypercalcemia, thus feed safety assessments are necessary. Recently, the tolerance of this product was assessed in growing chicken and could be fed up to 40 μg/kg without observed negative incidences (Mathis et al., 2016).

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The aim of this study was to determine the tolerance of growing pigs fed *Solanum glaucophyllum* leaves with a standardised content $1,25(OH)_2D_3$ in diets containing the maximal authorized supply of vitamin D_3. In addition, the recommended vitamin D level was compared with the maximal authorized level, without *Solanum glaucophyllum* leaves.

2. Material and methods

2.1. Experimental diets

Six experimental diets (Table 1) were formulated to meet or exceed all nutrient requirements of weaned pigs weighing 15 kg (Agroscope, 2006). The diets were formulated to contain, per kg, 9.7 g Ca and 3.5 g digestible P (dP) including 500 FTU of exogenous phytase (0.16 g dP/100 FTU, Natuphos®, BASF, Germany). One diet (1000/0) was supplemented with a premix (Protector, Lucens, Switzerland) to provide the recommended D_3 supply (Agroscope, 2006) of 1000 IU (25 μg) vitamin D_3/kg diet (Zhejiang Garden Biochemical High-Tech, Hangzhou, China). Five diets were supplemented with the same premix, with additional vitamin D_3 to provide 2000 IU (50 μg) vitamin D_3/kg diet. The level of 2000 IU D_3/kg corresponds to the maximal authorized cholecalciferol supplementation in European pig diets (EC, European Community, 2004). These five diets (2000/0, 2000/2.5, 2000/5, 2000/10 and 2000/20) were supplemented, per kg, with either 0, 2.5, 5, 10 and 20 μg $1,25(OH)_2D_3$ glycosides contained in a standardised formulation of dried *Solanum glaucophyllum* leaves (Panbonis®, Herbonis Animal Health, Augst, Switzerland). The studied product contained 10 mg of analytically determined $1,25(OH)_2$ D_3/kg and is, according to the supplier, recommended being added at 100 g/t complete feed to provide 1 μg $1,25(OH)_2$ D_3/kg diet on top of the usual supplemented cholecalciferol level. The experimental diets thus contained up to 20 times the recommended supplementation level in a diet containing the maximal authorized cholecalciferol content. Each experimental diet included differently coloured maize cob pellets to allow visual identification. All diets were produced at the Agroscope experimental feed mill and were pelleted (60 °C, 4 mm diameter).

2.2. Animals and experimental procedures

The experiment was approved by the animal welfare department of the competent government authority (approval n° FR 41/09 E). Sixty Large White pigs from the Agroscope breeding herd were weaned at 30 ± 5 days and at 9.2 ± 1.3 kg BW, and were assigned to ten blocks of six animals according to their BW and gender (female and castrated males). Within each block, the pigs were randomly allocated to the treatments and placed into one of the 12 pens for 44 days. The pens (4.5 m^2 each) were in one room and did not give outdoor access to the animals. Pigs had ad libitum access to the experimental diets, to tap water provided by nipple drinkers and to straw which was available in racks. At the end of the experiment, pigs from the diets 1000/0, 2000/0, 2000/10 and 2000/20 ($n = 40$) were stunned using CO_2 followed by exsanguination at the Agroscope research abattoir located on the experimental site.

2.3. Data and sample collection and analysis

Feed intake (pen basis) and BW (individual basis) were recorded on a weekly basis. Diet samples were collected weekly, pooled per diet and subsequently milled using a 1.0 mm screen (Brabender, Duisburg, Germany) before analysis. Blood was collected between 09 h00 and 10 h00 by jugular venepuncture from each pig at the beginning of the experiment and then on a weekly basis. Blood was transferred to lithium heparin tubes, put on ice and centrifuged (1600 ×*g*, 15 min) within 1 h after collection. Plasma samples were stored at −20 °C until analysis. At evisceration a urine sample was collected from the urinary bladder and the left kidney was collected. The urine samples were frozen (−20 °C) and the kidneys were cut into cubes of 1 cm^3, rinsed with a 0.9% NaCl solution and lyophilised. The left tibiae and the left 3rd metacarpal bones (Mc) were collected within half a day after slaughter. The tibiae were cleaned from soft tissues and all bones were stored in sealed plastic bags at −20 °C. The Mc were autoclaved (121 °C, 1 bar, 45 min), cleaned of soft tissues and their density was determined using the Archimedes principle according to Ogunbameru et al. (1990). The Mc were then crushed, defatted with acetone, dried overnight at 60 °C and milled using a 1.0 mm screen (Grindomix, Retsch, Haan, Germany) before chemical analysis. The tibiae were transferred from the freezer to a refrigerator 18 h before their breaking strength was determined using the three-point bending test (Zwick Roell, Ulm, Germany). The bones were held by two supports spaced 49 mm apart and were broken by a wedge lowered on the centre of the bone at a speed of 2 mm/s. The force was measured by a pressure-sensitive cell at the peak of force required before bone breaking.

2.4. Chemical analysis of samples

Dry matter (DM) content was quantified thermogravimetrically by heating at 105 °C for 3 h (Leco TGA 601, Mönchengladbach, Germany), and ash content was subsequently determined after incineration at 550 °C until constant weight was attained. Crude protein content in diets was determined as 6.25 ∗ nitrogen, where nitrogen was determined using the Kjeldahl procedure (AOAC, 1995). Crude fiber content in diets was determined after the samples were digested with successively H_2SO_4 and KOH, washed with acetone, dried at 130 °C and finally ashed (VDLUFA, 1976). Crude fat content in diets was determined as petrol ether extract after an acidic hydrolysis in boiling HCL for 1 h (VDLUFA, 1976). Dry ashed feed, kidney and Mc were solubilized with nitric acid and their mineral concentration was analysed according to the European Standard EN 155510:2008 using an inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 7300 DV, Perkin-Elmer, Schwerzenbach, Switzerland). Phytase activity in the diets was measured photometrically at 415 nm after incubation in a sodium phytate solution (ISO 30024). The plasma analytes were assayed using commercially available kits according to manufacturer's instruction on a Lisa 200 autoanalyser (Biomérieux, Marcy l'Etoile, France) to determine Ca and creatinine (Roche, Basel, Switzerland), phosphorous and magnesium (Biomérieux, Marcy l'Etoile, France) concentrations. Concentration of $1,25(OH)_2D_3$ was determined in the *Solanum glaucophyllum* leaves after its $1,25(OH)_2D_3$-glycosides extraction with ethanol/water and in the plasma from pen based pooled samples using an ELISA kit (Kit 2112, Immundiagnostik, Bensheim, Germany). The ELISA kit consists of an extraction step of the vitamin D metabolites, followed by a chromatographic separation of $1,25(OH)_2D_3$ from $25(OH)D_3$. The extracted $1,25(OH)_2D_3$ was then quantified by a competitive immune assay type with a specific antibody against $1,25(OH)_2D_3$. The assay characteristics for this ELISA kit are provided by the supplier as 6.7% intra- and 9% inter-assay precision and as a cross-reactivity of 41% against $1,25(OH)_2D_2$ and <0.1% against vitamin D_3, D_2, $25(OH)D_3$ and $25(OH)D_2$.

2.5. Statistical analysis

Plasma concentrations were analysed with the mixed model of SYSTAT 13 (SYSTAT Software, Inc.) including block, treatment, time and treatment × time. Initial concentrations were included as covariables. Final plasma concentrations and the other physiological traits provided from the killed pigs (only treatments 1000/0, 2000/0, 2000/10 and 2000/20) were analysed using the general linear model of SYSTAT 13 (2009) and included block and treatment. Slaughter weight was used as covariable for bone breaking strength. Differences were considered significant when $P < 0.05$ and trends were noted at $P < 0.10$.

3. Results

3.1. Diets and animal performance

The analysed nutrient contents of the experimental diets matched the expected values (Table 1). Calcium and P contents and phytase activity were respectively 9.7 ± 0.2 and 5.5 ± 0.1 g/kg and 705 ± 63 FTU/kg (mean ± standard deviation).

During the first 2 weeks of the experiment, 13 of the 60 pigs were treated against diarrhoea with sulphonamide/trimethoprim administered by injection and all animals finished the experiment in good health. The daily intake of $1,25(OH)_2D_3$ from the tested product (Fig. 1) increased linearly between the first and the last week of the experiment from 0.04 to 0.15, from 0.08 to 0.30, from 0.17 to 0.61 and from 0.46 to 1.22 μg/kg BW in, respectively, 2000/2.5, 2000/5, 2000/10 and 2000/20. The average final BW, daily BW gain, daily feed intake and feed conversion ratio were 25.4 ± 4.9 kg, 378 ± 100 g, 623 ± 68 g and 1.65 ± 0.11, respectively.

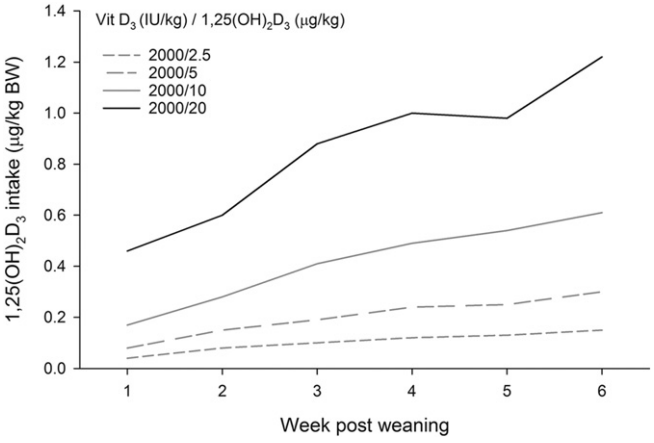

Fig. 1. Daily intake of $1,25(OH)_2D_3$ from the supplemented standardised formulation of dried *Solanum glaucophyllum* leaves (μg per kg body weight).

3.2. Plasma parameters

The initial Ca, P and $1,25(OH)_2D_3$ plasma values were similar ($P > 0.10$) between dietary treatments. Over the six weeks, plasma Ca (Fig. 2) and plasma P (Fig. 3) increased ($P < 0.001$) by respectively 16 and 27% and plasma $1,25(OH)_2D_3$ (Fig. 4) only tended to increase ($P = 0.07$). Twenty μg of $1,25(OH)_2D_3$ resulted in higher plasma Ca from the 2nd experimental week onwards (Treatment × Time interaction, $P < 0.05$) and maintained Plasma P constant (Treatment × Time interaction, $P < 0.001$) from the 4th week onwards. Final plasma concentrations of 1000/0, 2000/0, 2000/10 and 2000/20 are presented

Table 1
Formulation and composition of the experimental diets (as fed basis).

Treatment	1000/0	2000/0	2000/2.5	2000/5	2000/10	2000/20
Ingredients (g/kg)						
Maize	420	420	420	420	420	420
Barley	250	250	250	250	250	250
Oat flakes	30	30	30	30	30	30
Wheat middlings[a]	4	4	4	4	4	4
Expelled soybean meal	70	70	70	70	70	70
Casein powder	60	60	60	60	60	60
Whey powder	50	50	50	50	50	50
Maize gluten	10.6	10.6	10.6	10.6	10.6	10.6
Apple pomace	50	50	50	50	50	50
Fat (tallow and lard)	10	10	10	10	10	10
Calcium formate	10	10	10	10	10	10
Calcium carbonate	6	6	6	6	6	6
Dicalcium phosphate	10.6	10.6	10.6	10.6	10.6	10.6
NaCl	3	3	3	3	3	3
L-lysine HCl	2.1	2.1	2.1	2.1	2.1	2.1
L-threonine	0.6	0.6	0.6	0.6	0.6	0.6
Coloured maize cob[b]	6.0	6.0	6.0	6.0	6.0	6.0
Binder[c]	3.0	3.0	3.0	3.0	3.0	3.0
Vit./min. premix 1000[d]	4.0					
Vit./min. premix 2000[e]		4.0	4.0	4.0	4.0	4.0
Phytase[f]	0.1	0.1	0.1	0.1	0.1	0.1
Nutrient composition						
Digestible energy (MJ/kg)[h]	14.0	14.0	14.0	14.0	14.0	14.0
Crude protein (g/kg)[g]	169	173	175	173	174	173
Fat (g/kg)[g]	55	57	61	58	55	58
Crude fiber (g/kg)[g]	35	39	35	38	37	36
Ash (g/kg)[g]	54	55	55	54	55	56
Ca (g/kg)[g]	9.7	9.8	9.7	9.3	9.8	9.8
P (g/kg)[g]	5.3	5.5	5.7	5.5	5.5	5.4
Phytase act. (FTU/kg)[g]	800	680	750	680	700	620
Digestible P (g/kg)[h]	3.5	3.5	3.5	3.5	3.5	3.5

1000/0 = 1000 IU D_3/kg; 2000/0 = 2000 IU D_3/kg; 1000/2.5 = 2000 IU D_3/kg and 2.5 μg $1,25(OH)_2D_3$/kg; 1000/5 = 2000 IU D_3/kg and 5 μg $1,25(OH)_2D_3$/kg; 1000/10 = 2000 IU D_3/kg and 10 μg $1,25(OH)_2D_3$/kg; 1000/20 = 2000 IU D_3/kg and 20 μg $1,25(OH)_2D_3$/kg.

[a] Supplemented with $1,25(OH)_2$ D_3 (Panbonis®, Herbonis, Basel, Switzerland) to provide either 0, 2.5, 5, 10 and 20 μg/kg diet.

[b] Microgrit, Microtracers, San Francisco, U.S.A.

[c] Pellan, Mikro-Technik, Bürgstadt, Germany.

[d] Supplied per kg of diet: 40 mg Fe; 0.15 mg I; 6 mg Cu; 10 mg Mn; 75 mg Zn; 0.2 mg Se; 8000 IU vitamin A; 1000 IU vitamin D_3; 25 mg vitamin E; 3 mg vitamin K_3; 2 mg thiamine; 5 mg riboflavin; 0.1 mg biotin; 20 mg niacin; 15 mg pantothenic acid.

[e] As Vit./min. premix 1000, but supplied 2000 IU vitamin D_3/kg diet, instead of 1000 IU vitamin D_3/kg diet.

[f] Natuphos® 5000 G, BASF, Ludwigshafen, Germany, 5000 FTU/g.

[g] Analysed as described under section Material and methods.

[h] Calculated according to Agroscope (2006).

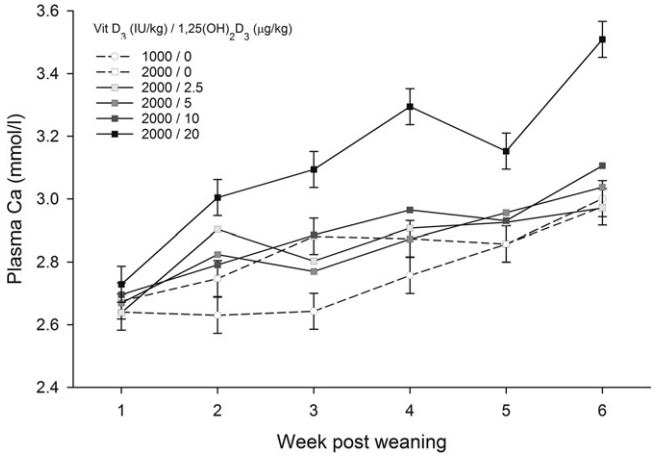

Fig. 2. Effect of dietary vitamin D_3 and 1,25 dihydroxyvitamin D_3 glycosides on plasma Ca evolution. Probability levels of variables were: dietary treatments ($P < 0.001$), time ($P < 0.001$) and treatment × time interaction ($P < 0.05$). Standard errors illustrated for 1000/0, 2000/0 and 2000/20.

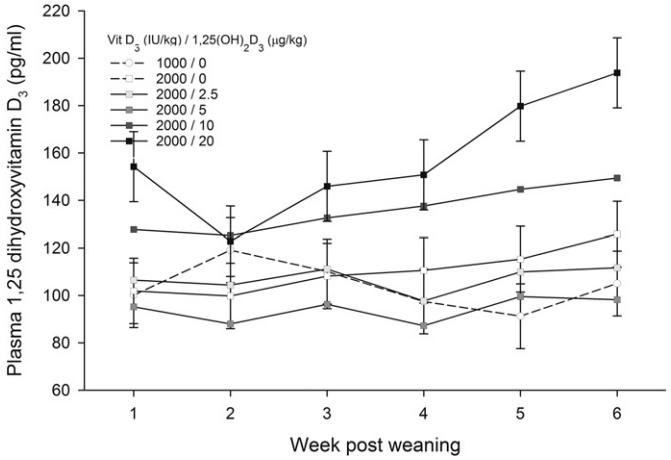

Fig. 4. Effect of dietary vitamin D_3 and 1,25 dihydroxyvitamin D3 glycosides on plasma $1,25(OH)_2D_3$ evolution. Probability levels of variables were: dietary treatments ($P < 0.001$), time ($P = 0.07$) and treatment × time interaction ($P > 0.10$). Standard errors illustrated for 1000/0, 2000/0 and 2000/20.

in Table 2. Final plasma Ca was higher ($P < 0.001$) in 2000/20 compared to the other treatments and represented an increase of 19% when compared with 2000/0. Final plasma P decreased by 16% ($P < 0.05$) in 2000/20 compared to 1000/0. Final plasma $1,25(OH)_2D_3$ tended to be increased by 84 and 56% with 2000/20 compared to 1000/0 and 2000/0, respectively. Final plasma Ca, P and $1,25(OH)_2D_3$ remained similar ($P > 0.10$) between 1000/0 and 2000/0.

3.3. Kidney, urinary and bone parameters

The urinary, kidney and Mc mineral concentrations and the physical bone parameters are presented in Table 2. Urinary P was low and was not influenced ($P > 0.10$) by dietary treatments, but urinary Ca tended to be increased ($P = 0.06$) by 69% in 2000/20 in reference to 2000/0 and to 1000/0. Kidney Ca and P concentrations were influenced ($P < 0.01$) by the dietary treatments where Ca concentration in 2000/20 was increased by 43% compared to the other treatments and where P concentration was higher in 2000/10 compared to 1000/0 and to 2000/20. Metacarpal ash and P contents in 2000/20 tended to be reduced ($P = 0.09$ and $P = 0.06$, respectively) by respectively 3 and 4% compared to the other treatments. Metacarpal Mg in 2000/20 was reduced ($P < 0.05$) by 8% compared to 1000/0. Bone density was not influenced ($P > 0.10$) by dietary treatments, but bone breaking strength in 2000/20 was deteriorated ($P < 0.05$) by 12% compared to 2000/0. Urine, kidney and bone parameters remained similar ($P > 0.10$) between 1000/0 and 2000/0.

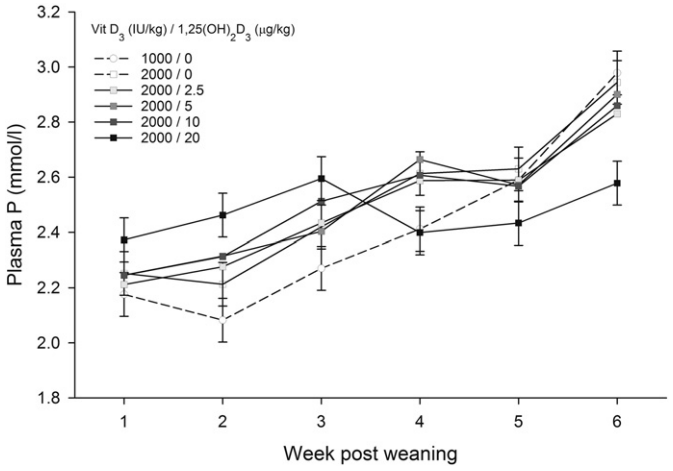

Fig. 3. Effect of dietary vitamin D_3 and 1,25 dihydroxyvitamin D_3 glycosides on plasma P evolution. Probability levels of variables were: dietary treatments ($P > 0.10$), time ($P < 0.001$) and treatment × time interaction ($P < 0.001$). Standard errors illustrated for 1000/0, 2000/0 and 2000/20.

4. Discussion

Feed consumption and consequently the intake of 1,25 $(OH)_2D_3$ per kg BW increased about threefold between the first and the sixth week after weaning. The continuous increase in plasma P and, to a lower extent, in plasma Ca reflects the increasing nutrient intake. Hypercalcemia is considered to be the most sensitive clinical chemical parameter of an excessive 1,25 $(OH)_2D_3$ intake, because the principal function of 1,25 $(OH)_2D_3$ is to promote intestinal Ca absorption in order to maintain plasma Ca within physiological limits. Plasma Ca values exceeding the upper normal limit of 2.9 mmol/l (Kaneko et al., 2008) were observed

Table 2
Effect of dietary vitamin D_3 and 1,25 dihydroxyvitamin D_3 glycosides on mineral status at the end of experiment.

	Treatment				SEM[a]	*P* value[a]
	1000/0	2000/0	2000/10	2000/20		
Plasma (mmol/l)[b]						
Ca	2.99^{x}	2.96^{x}	3.10^{x}	3.51^{y}	0.07	***
P	2.98^{y}	2.95^{xy}	2.86^{xy}	2.58^{x}	0.10	*
$1,25(OH)_2D_3$	112^{X}	132^{X}	155^{XY}	206^{Y}	16	0.06
Urine						
Creatinine (mmol/l) [c]	5.26	5.42	3.93	3.13	1.36	n.s.
Ca (mol/mol creatinine)[c]	3.96^{X}	3.98^{X}	4.69^{XY}	6.70^{Y}	0.72	0.06
P (mol/mol creatinine) [c]	0.10	0.08	0.19	0.20	0.06	n.s.
Kidney (g/kg DM)						
Ca	0.50^{x}	0.51^{x}	0.51^{x}	0.73^{y}	0.05	**
P	12.3^{x}	12.8^{xy}	13.0^{y}	12.2^{x}	0.2	**
Mc (g/kg DM)						
Ash	582^{Y}	580^{Y}	582^{Y}	564^{X}	5	0.09
Ca	231	232	233	230	3	n.s.
P	117^{XY}	118^{Y}	118^{Y}	113^{X}	1	0.06
Mg	4.48^{y}	4.39^{xy}	4.24^{xy}	4.13^{x}	0.09	*
Mc Density (g/cm^3)	1.06	1.09	1.06	1.07	0.02	n.s.
Tibia breaking strength (N)	1724^{y}	1753^{y}	1656^{xy}	1541^{x}	48	*

1000/0 = 1000 IU D_3/kg; 2000/0 = 2000 IU D_3/kg; 1000/10 = 2000 IU D_3/kg and 10 µg $1,25(OH)_2D_3$/kg; 1000/20 = 2000 IU D_3/kg and 20 µg $1,25(OH)_2D_3$/kg.

[a] SEM = standard error of the mean; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; n.s. $P > 0.10$; values in a same row not followed by the same superscript letter differ significantly; values in a same row not followed by the same superscript capital letter differ with tendency.

[b] Collected prior slaughter.

[c] Urine from urinary bladders was successfully collected from 5 pigs per treatment.

from the 3rd week onwards in the pigs fed 2000/20, when their daily 1,25 $(OH)_2D_3$ intake was between 0.9 and 1.2 μg per kg BW. This dose can therefore be considered as potentially toxic, because a chronic plasma Ca concentration > 3.0 mmol/l can cause soft tissue calcification (NRC, National Research Council, 2005).

Plasma P remained within the reference range for pigs of 1.7–3.1 mmol/l (Kaneko et al., 2008). During the first three weeks, plasma P was however below the concentration of 2.5 mmol/l considered necessary for maximum growth (Suttle, 2010). Plasma P of pigs fed 2000/20 was highest during the first three weeks, but was lowest and still below 2.5 mmol/l afterwards when plasma Ca exceeded 3 mmol/l. A decrease in plasma P concentration was previously reported in weaned pigs fed *Solanum glaucophyllum* (Rucksan et al., 1978), whereas hyperphosphatemia in addition to hypercalcemia were described in calves, sheep, rabbits and guinea pigs fed this plant (Döbereiner et al., 1975; Camberos et al., 1969, 1970; Mautalen, 1972). The reason for a decreased plasma P in pigs with hypercalcemia remains unclear, because the perfusion of *Solanum glaucophyllum* extract increased the intestinal absorption of P, as well as, of Ca in pigs (Fox and Care, 1979) and because the urinary P/creatinine ratio did not differ between the treatments in the present experiment, suggesting that urinary P excretion was similar in all groups of pigs. Since a high plasma P concentration promotes the risk of soft tissue calcification (Giachelli, 2009), the comparably low sensitivity of pigs to soft tissue calcification (Weissenberg, 1989) may be due to the plasma P lowering effect of *Solanum glaucophyllum* in this animal species.

Although the final plasma 1,25 $(OH)_2D_3$ concentration in pigs fed 2000/20 was almost twice as high as in the two unsupplemented animals, higher concentrations were detected in pigs fed balanced diets without supplementation of 1,25$(OH)_2D_3$ (Engstrom et al., 1985). The uptake of ingested 1,25$(OH)_2D_3$ by the intestinal mucosa directly stimulates intestinal Ca absorption (Massheimer et al., 1994). Hypercalcemia is therefore a more reliable in vivo indicator of an excessive intake of 1,25$(OH)_2D_3$ in pigs than the plasma level of that metabolite itself.

The post mortem data confirm the evaluation based on plasma Ca and P concentration that the tolerance was exceeded when 2000/20 was fed. Kidney Ca concentration increased in 2000/20, but was still within the physiological range of <0.8 g/kg DM (NRC, National Research Council, 2005). As a comparison, Pointillart et al. (1978) determined that 7 g Ca/kg DM was indicative for nephrocalcinosis in the kidneys of growing pigs fed a diet excessive in P (12 g P/kg). The increased urinary Ca in 2000/20 illustrates that the homeostatic regulation aimed to maximize Ca excretion. The highest dietary level of 1,25$(OH)_2D_3$ glycosides also affected the bone metabolism of the growing animals, as shown by the reduced bone breaking strength and the tendency for a reduced bone ash concentration. Previously, *Solanum glaucophyllum* caused hyperostosis and a thinner cortical bone in pigs (Done et al., 1976), which reduces the mechanical force of the bone.

Since the experimental diet was fed dry, the 1,25$(OH)_2D_3$ glycosides were hydrolysed by glycosidase present in the gastrointestinal tract of the pigs. Incubation of *Solanum glaucophyllum* with rumen fluid prior to the administration to pigs increased its hypercalcemic effect (Rucksan et al., 1978), presumably because of extensive cleaving of the glycoside by the rumen microbes. Because the microbial count in liquid feed for pigs is quite high (Brooks et al., 2001), *Solanum glaucophyllum* administered in liquid feed may show a higher activity and consequently a lower tolerance than reported here.

Finally, the present data also imply that the increase in dietary D_3 supply to the maximal authorized level of 2000 IU/kg did not bring any benefits to the pigs during the post weaning period when compared to the recommended level of 1000 IU/kg.

5. Conclusions

Based on the results of this six week tolerance study, the addition of up to 10 μg 1,25 dihydroxyvitamin D_3 glycosides/kg diet (10 times the manufacturer's recommended level) did not cause any adverse effects in growing pigs. No soft tissue calcification is to be expected in growing pigs fed up to 10 μg 1,25$(OH)_2D_3$ glycosides/kg in a diet containing the recommended amounts of Ca and dP and the maximal authorized vitamin D content. The addition of 20 μg/kg diet of 1,25$(OH)_2D_3$ glycosides (20 times the manufacturer's recommended level) resulted in a daily intake of about 1 μg 1,25$(OH)_2D_3$ per kg BW during the second half of the experiment and caused adverse effects such as hypercalcemia, a decreased bone mineralization and bone strength. Finally, no benefits were observed when 2000 IU vitamin D_3/kg diet was fed instead of 1000 IU.

Conflicts of interest

None.

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