25-Hydroxyvitamin D₃ affects vitamin D status similar to vitamin D₃ in pigs – but the meat produced has a lower content of vitamin D

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In food databases, the specific contents of vitamin D₃ and 25-hydroxyvitamin D₃ in food have been implemented in the last 10 years. No consensus has yet been established on the relative activity between the components. Therefore, the objective of the present study was to assess the relative activity of 25-hydroxyvitamin D₃ compared to vitamin D₃. The design was a parallel study in pigs (n 24), which from an age of 12 weeks until slaughter 11 weeks later were fed approximately 55 μg vitamin D/d, as vitamin D₃, in a mixture of vitamin D₃ and 25-hydroxyvitamin D₃, or 25-hydroxyvitamin D₃. The end-points measured were plasma 25-hydroxyvitamin D₃, and in the liver and loin the content of vitamin D₃ and 25-hydroxyvitamin D₃. Vitamin D₃ and 25-hydroxyvitamin D₃ in the feed did not affect 25-hydroxyvitamin D₃ in the plasma, liver or loin differently, while a significant effect was shown on vitamin D₃ in the liver and loin (P<0·001). 25-Hydroxyvitamin D₃ in the plasma, liver and loin significantly correlates with the sum of vitamin D₃ and 25-hydroxyvitamin D₃ in the feed (P<0·05). Therefore, 25-hydroxyvitamin D₃ should be regarded as having the same activity as vitamin D₃ in food databases. Sole use of 25-hydroxyvitamin D₃ as a vitamin D source in pig feed will produce liver and meat with a negligible content of vitamin D₃, while an increased content of vitamin D₃ in the feed will produce liver and meat with increased content of both vitamin D₃ and 25-hydroxyvitamin D₃.

25-Hydroxyvitamin D₃: Vitamin D₃: Pig feed: Activity: Status

Vitamin D deficiency increases the risk of bone fracture due to osteoporosis and decreases muscle strength. Recent investigations show a relationship between vitamin D deficiency and other afflictions such as cancer, reduced immune defence and CVD¹³⁻¹⁵. During the summer period, the primary source of vitamin D for man exposed to sunlight is the metabolism of 7-dehydrocholesterol to pre-vitamin D₃ in the skin by UV B radiation (290–315 nm), whereas vitamin D in food is the secondary source. In winter oral intake of vitamin D may be the primary source, as absorption through the skin is limited at latitudes above 35°, e.g. for 4 months in Boston, USA (42°N) and for 6 months in Bergen, Norway (61°N)³. Similarly, oral intake of vitamin D is the primary source all year round for people not exposed to sunlight due to confinement indoors or clothing.

Estimation of dietary intake of vitamin D is essential for investigating the influence of vitamin D on health parameters in a population as well as in human intervention studies. Such calculations are based on dietary intake data from dietary surveys combined with the content of nutrients available in food composition tables. Until 10–15 years ago vitamin D data in food composition tables was mainly derived from biological assays, which used the ability of vitamin D to cure rickets in vitamin D-deficient rats⁴,⁵. For the last 10 years, food composition tables have included specific values for vitamin D₃ (vitD₃) and 25-hydroxyvitamin D₃ (25OHD₃)⁷⁻¹². To calculate the total vitamin D content, the relative activity between 25OHD₃ and vitD₃ is required.

The studies conducted to assess this factor were performed 30–40 years ago in deficient rats, in which the estimated values were between 1·4 and 5¹³⁻¹⁶. However, to date, no consensus has been established¹⁷.

The aim of the present study was to investigate the relative activity between vitD₃ and 25OHD₃ in pigs, as a model for man. The end-points were plasma 25OHD₃, and the content of vitD₃ and 25OHD₃ in the pork loin and liver. Vitamin D₃ (vitD₃) throughout the paper is defined as the sum of vitD₃ and 25OHD₃.

Materials and methods

Pigs

The twenty-four pigs selected for the present study were a subgroup of 3225 healthy pigs used in a feeding-trial conducted to investigate whether the productivity of the pigs was affected when vitD₃ was replaced by 25OHD₃. The feeding-trial was conducted at an ordinary Danish farm in stables with partially slatted floors and cover. A computer-controlled system

Abbreviations: 25OHD₃, 25-hydroxyvitamin D₃; vitD₂, vitamin D₂; vitD₃, vitamin D₃.
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distributed the feed to the separate double pens. Each double pen contained forty-five pigs. The pigs were fed via a tube feeder with nipple drinks as well as drinking bowls. The pigs were raised without exposure to sunlight. The stable lighting was produced with lamps (F36W/T8/33-630; Osram, Sylvania, MA, USA).

Experimental design

The study was performed as a supplementation study and consisted of a parallel trial with three treatments of vitamin D. Vitamin D was given at equal levels as vitD₃, as a mixture of vitD₃ and 25OHD₃, or as 25OHD₃, from weaning at an age of 5 weeks to slaughter at an age of approximately 5.5 months. The feedstuff used was produced by DSM Nutritional Products (Copenhagen, Denmark) and DLG (Danske Landbrugs Grovvaeselskab, Copenhagen, Denmark). Detailed information of the content of the feed is given elsewhere.

Sampling

The amount fed to each pig was calculated per pen for each of the three periods: 6–7 weeks, 8–12 weeks, 13–23 weeks (weight of 32.5 kg, and until slaughter at approximately 100 kg). For the same periods feed was sampled under the principle of the theory of sampling, and analysed for vitD₃ and 25OHD₃.

The day before slaughter a blood sample was drawn, and processed to EDTA–plasma and stored at −80°C until analysis. At slaughter the liver was sampled and the next day boneless loin with rind was separated. The loin was subsequently carefully dissected into lean meat without any subcutaneous fat (lean meat), subcutaneous fat without any lean meat (fat), and skin without any subcutaneous fat (skin). All the samples were packed in plastic bags and frozen at −20°C until analysis, which was performed within 8 months. Before analysis the liver, lean meat and fat were slowly thawed and separately ground in a homogenizer (1094 Homogenizer; Tecator, Paris, France) for 2 min, while the skin was slowly thawed and manually cut into pieces of 10–15 mm².

Vitamin D₃ and 25-hydroxyvitamin D₃ in the feed

DSM Laboratory (Basel, Switzerland) carried out analyses of vitD₃ and 25OHD₃ in the feed. For quantification of 25OHD₃, 10 g feed was added to 500 ng d₆-hydroxyvitamin D₃ (synthesized by Prof. Mourino, University of Santiago de Compostela, Spain) as the internal standard, and 60 ml water. The sample was gently swirled into a slurry and sonicated at 50°C for 10 min. The vitamins were extracted with 40 ml tert-butyl methyl ether by shaking and sonication for 5 min followed by centrifuging for 3 min at 3000 rpm. Supernatant (10 ml) was evaporated and the residue dissolved in 2 ml mobile phase for preparative HPLC (2-propanol–ethanol–isooctane, 1:10:89). For clean-up 100 µl were injected into a preparative HPLC system equipped with a silica-column (Sí60, 3 µm, 150 × 4.6 mm; Hypersil, Shandon Products, Runcorn, UK). The fraction of 25OHD₃ and the internal standard with a retention time of 14–16 min was collected. Subsequently, the organic solvent was evaporated and dissolved in 700 µl methanol and 300 µl water. The quantitative determination was performed by injection of 90 µl into the HPLC–atmospheric pressure chemical ionization–MS equipment (Agilent 1946C LC/MSD single-quadrupole mass specific detector equipped with an atmospheric pressure chemical ionization unit; Agilent Technologies AG, Basel, Switzerland). Additionally, the HPLC system consisted of a C18 column (Aquasil C18 (Aquasil, Thermo Fisher Scientific, Waltham, MA, USA), 3 µm, 20 × 100 mm) and the mobile phase was a gradient of methanol–water (99:95:0.5).

The quantification of vitD₃ in the feed was determined by using vitamin D₂ (vit D₂) as the internal standard according to EN12821.

Vitamin D₃ and 25-hydroxyvitamin D₃ in the meat

The analytical method and the equipment used to determine vitD₃ and 25OHD₃ in the meat are previously described. Minor modifications were made as 25-hydroxyvitamin D₂ (Sigma-Aldrich, Buchs, Switzerland) was used as the internal standard for 25OHD₃ similar to the utilization of vitD₃ as the internal standard for vitD₃. Briefly, the internal standards of vitD₂ and 25-hydroxyvitamin D₂ were added to the meat samples and saponified with ethanolic potassium hydroxide. The unsaponifiable matter was extracted with diethyl ether–petroleum ether (1:1). The solution was then purified on a silica solid-phase extraction column and further cleaned by preparative HPLC equipped with silica and amino columns. Analysis of the liver samples included an extra preparative HPLC procedure, which consisted of a cyano column (Luna, Cyan 5µm, 150 ×4.6 mm) from Phenomenex (Torrance, CA, USA), and a mobile phase of 2-propanol–n-heptane (1.5:98.5). The fraction of vitD₂ and vitD₃ co-eluted with a retention time of 5 min at a flow rate of 1 ml/min. This fraction was collected and evaporated to dryness using a gentle stream of nitrogen, and finally dissolved in methanol–acetonitrile (20:80). Another fraction containing 25-hydroxyvitamin D₂ with retention time at 16 min as well as 25OHD₃ with retention time at 21 min was collected in the same vial, evaporated and dissolved in acetonitrile–water (90:10). These two fractions were injected into the analytical HPLC system described earlier.

Content of fat in the meat

Content of fat in the meat was determined by the gravimetric method following a modified Schmid–Bondzynski–Ratslaff method. Briefly, the sample was boiled with hydrochloric acid followed by the addition of ethanol and extraction of the lipids with diethyl ether–petroleum ether (1:1). After evaporation of the solvent, the fat was weighed.

Plasma 25-hydroxyvitamin D

The quantification of 25OHD₃ in plasma was performed by the HPLC method described previously. Briefly, plasma proteins were precipitated with ethanol and the supernatant was cleaned by a MFC18 solid-phase extraction. The 25OHD₃ in the solution was separated, detected and measured by analytical HPLC equipped with a diode array detector (220–320 nm) and a UV detector (265 nm) and external calibration.
Data analysis

Based on previously assessed variation of content of vitD3 and 25OHD3 in meat, six pigs should be included in each feeding group to detect a relative activity of 1.5 for 25OHD3 compared to vitD3 with a power of 80% and a significance level of 5%.

To test the effect of the content of 25OHD3 and vitD3 in feed on 25OHD3 in plasma and on 25OHD3 and vitD3 in meat and liver, regression analysis was performed. In the regression model, 25OHD3 in plasma, meat and liver, and vitD3 in meat and liver were dependent variables, and the total content of vitamin D in feed (vitamin Dtotal) and the difference between 25OHD3 and vitD3 (vitamin Ddiff) were independent variables. Furthermore, ANOVA was performed with feed as an independent fixed variable to test and estimate differences between feeding groups. Association between determinants and variables were assessed with Pearson’s correlation coefficients. Data are expressed as means and their standard errors. SAS version 9.1 (SAS Institute, Cary, NC, USA) was used for all statistical analyses, with a significance level of 0.05.

Results

Pigs performance

The vitamin D in each of the three diets was given as the same vitamin D source(s) but due to the different feed and consumption levels during the growth period, the mean daily intake differed. vitD3 and 25OHD3 in each of the three feeding periods are presented in Fig. 1. Carcass weights are presented in Table 1. No significant difference was detected between the diets or between the subgroup and the pigs included in the main feeding study (n 3225).

Effect of 25-hydroxyvitamin D3 and vitamin D3 as vitamin D source

Results for vitamin D status assessed as plasma 25OHD3, and the contents of 25OHD3 and vitD3 in the liver, and in the three separated parts of the loin including the content of fat are presented in Table 1.

The results show that 25OHD3 in the plasma, in the liver and in the three separated parts of loin did not depend on the vitamin D source, but on the daily intake of vitamin Dtotal, as content of 25OHD3 in the separated cuts was significantly associated with vitamin Dtotal (R 0.42–0.56; P<0.05).

In contrast, vitD3 in the liver and in the three separated parts of loin depended on the vitamin D source (P<0.001), and showed no association with vitamin Dtotal. However, content of vitD3 was significantly associated with vitD3 in the feed (R 0.65–0.89; P<0.001).

Discussion

Previous work has shown that vitD3 and 25OHD3 in meat is positively associated with the content of fat. Therefore it is essential that comparison of meat derived from pigs fed different diets does not differ in the content of fat. No significant difference was shown for subcutaneous fat, lean meat and skin. The present results show that the intention to produce three similar separated cuts from each pig was fulfilled regarding the content of fat.
The significant effect of the feeding level of vitamin D total on plasma 25OHD3 in these pigs was in line with the positive association between dietary intake of vitamin D total and serum 25OHD3 shown in women and men.\(^{15,26}\)

The present study was originally designed to investigate whether the productivity of the pigs was affected when the vitD3 was replaced by 25OHD3 in the feed. No difference was found in the present study which included 3225 pigs.\(^{18}\) As the twenty-four pigs selected for the present nutritional study did not differ from the whole group concerning weight, and growth rate, the mean daily intake of vitamin D calculated from the whole group is applied.

Plasma 25OHD determined as the sum of plasma 25-hydroxyvitamin D3 and plasma 25OHD3 in these pigs was in line with the positive association between dietary intake of vitamin D total and serum 25OHD3 shown in women and men.\(^{15,26}\)

The observed effect that plasma 25OHD3 reaches a steady state if supplemented at the same level for an adequate time period has previously been used to study differences between natural and synthetic vitD2, vitD3, and 25OHD3 in fortified bread, juice and supplement, as well as different levels of vitD3 supplement.\(^{25-28,30}\) In human intervention studies supplementation levels of 5–10 µg/d for 4 weeks was shown to be adequate to reach a steady state for vitamin D status (J Jakobsen, unpublished results). The half-life of vitamin D in man is 1 month.\(^{11}\) Though the differences in the rate of metabolism between pigs and man are unknown for vitamin D, the applied period of 16 weeks to reach a steady state is assumed to be adequate. As the metabolism in pigs and man regarding fat-soluble vitamins is rather similar, it is assumed that the vitamin D status and vitamin D in the liver and in the meat at slaughter was not influenced by the feed given in the earliest stage of growth up to an age of 12 weeks.

To our knowledge, this is the first study investigating the effect of 25OHD3 and vitD3 in healthy mammals. Thirty to 40 years ago the difference between 25OHD3 and its parent vitD2 was tested in vitamin D-deficient rats either by testing the effect on intestinal calcium absorption measured by the everted gut sac technique, serum calcium and body weight, or by the ability to cure rickets. By the everted gut sac technique an equal effect of the two compounds was shown after 24 h, though 25OHD3 acted more rapidly.\(^{13,32,33}\)

In 1973, 25OHD3 was shown to be five times as active as vitD3 in the maintenance of serum calcium and growth.\(^{10}\) In the ability to cure rickets 25OHD3 had an effect 1.4–2 times the activity of vitD3 in three different studies, but in another study the effect was estimated to be 5 times as active.\(^{13-16}\)

Today, the factor of 5 for the activity between 25OHD3 and vitD3 is widely used in recommended dietary allowances as well as its implementation in food composition tables.\(^{10-12}\) However, the documentation for the factor of 5 seems limited due to the non-standardized methods used.\(^{13,16}\)

The data obtained for the relative activity between 25OHD3 and vitD3 in pigs need to be verified in a similar study in man, as well as further investigation of the possible difference of the effect of vitamin D derived from pork and from supplements. For mushrooms, no difference was shown between natural vitD2 and vitD2 given as a supplement in the ability to increase vitamin D status, investigated in a human intervention study.\(^{28}\) However, the vitamin D activity in meat may not be reflected only by vitD3 and 25OHD3. The content of 1,25-dihydroxyvitamin D3 and other dihydroxyvitamin D3 compounds are unknown, but may contribute to the vitamin D activity of meat.

The effect of 25OHD3 and its parent form vitD3 on plasma 25OHD3 should be regarded equal in the diet for pigs. However, for the nutritional value of pork meat, 25OHD3 in pig feed should be regarded as rather low compared with vitD3, as the content of vitD3 depended on the vitamin D source. The use of 25OHD3 only in the feed instead of vitD3 produced meat and liver with significantly lower content of vitD3. That the pigs fed solely on 25OHD3 did not produce meat and liver with vitD3 is not surprising, as vitD3 is not synthesized in the pigs. The hydroxylation of vitD3 to 25OHD3 by 25-hydroxylase is not a reversible reaction.

Additionally, the present study shows that the concentration of vitD3 as a vitamin D source in the feed determines the concentration of vitD3 in meat and liver even at small differences in the feeding levels, which was previously shown in pigs fed super nutritional levels at 1000 µg vitD3/kg.\(^{15}\) Additional feeding trials are necessary to investigate fully the possibility of pork meat bio-fortified with vitamin D.

Presently, a human intervention study is being conducted to evaluate whether human subjects respond to supplements of vitD3 and 25OHD3 in a similar fashion to pigs. However, more research on the relative bioactivity of vitamin D total from animal products compared to supplements of vitD3 is an important issue for the calculation of dietary vitD intake.

**Conclusion**

The findings of the present study showed that 25OHD3 and vitD3 equally affect 25OHD3 in plasma, meat and liver.

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**Table 1.** Weight of the carcass and the effect of the diet groups on 25-hydroxyvitamin D3 (25OHD3) in the serum, liver and separated parts of the loin and vitamin D3 (vitD3) in the liver and separated parts of the loin at slaughter

\(^*\) Diets A, B and C are vitD3, a mixture of vitD3 and 25OHD3, and 25OHD3, respectively.

\(^{abc}\) Mean values within a row with unlike superscript were significantly different (P < 0.05).

Data obtained for the relative activity between 25OHD3 and vitD3 in pigs need to be verified in a similar study in man, as well as further investigation of the possible difference of the effect of vitamin D derived from pork and from supplements.
However, for the benefit of human nutrition, 25OHD₃ in pig feed should be regarded as lower than vitD₃, as meat and liver produced by feeding the pigs exclusively 25OHD₃ had a significantly lower content of vitD₃. Regardless of the vitamin D source the present study identified a dose–response effect between vitamin D₉ total in the meat and in the liver with vitamin D₉ total in the feed, which for meat and liver indicate the possibility to produce meat and liver bio-fortified with vitamin D.

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