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25-hydroxyvitamin D circulates in different fractions of calf plasma if the parent compound is vitamin D_2 or vitamin D_3, respectively

Lone Hymøller* and Søren K Jensen

Department of Animal Science, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark

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Vitamin D has become one of the most discussed nutrients in human nutrition, which has led to an increased interest in milk as a vitamin D source. Problems related to fortifying milk with synthetic vitamin D can be avoided by securing a high content of natural vitamin D in the milk by supplying dairy cows with sufficient vitamin D. However, choosing the most efficient route and form of supplementation requires insight into how different vitamin D metabolites are transported in the body of cattle. There are two forms of vitamin D: vitamin D_2 (D_2) and vitamin D_3 (D_3). Vitamin D_2 originates from fungi on roughage. Vitamin D_3 originates either from endogenous synthesis in the skin or from feed supplements. Vitamin D_2 is chemically different from, and less physiologically active than, D_3. Endogenous and dietary D_3 is chemically similar but dietary D_3 is toxic, whereas endogenous D_3 appears well regulated in the body.

Keywords: Calf, cattle, cholecalciferol, endogenous, ergocalciferol, lipoprotein, synthetic, vitamin D_2, vitamin D_3.

The hypotheses of this study are that the liver derived metabolites of vitamin D: 25-hydroxyvitamin D_2 ($25OHD_2$) and 25-hydroxyvitamin D_3 ($25OHD_3$) are transported in different plasma fractions, causing the difference in physiological efficiency between D_2 and D_3, and that $25OHD_3$ produced from D_3 of endogenous or synthetic origin, respectively, are transported in different plasma fractions, contributing to their difference in toxicity.

Plasma from six calves was fractionated by ultracentrifugation into five fractions: chylomicron, lipoprotein fraction (LPF) 1, LPF2, LPF3, and protein. Samples were subsequently analysed for contents of $25OHD_2$ and $25OHD_3$. Results showed that $25OHD_3$ was transported in the protein fraction regardless of its origin, whereas $25OHD_2$ was transported in chylomicron and LPF2/LPF3 fractions. Associating with designated binding proteins is vital to the function and stability of $25OHD_2$ and $25OHD_3$, hence transport in other fractions than protein may explain the reduced circulating levels of $25OHD_2$ compared to $25OHD_3$, when D_2 or D_3, respectively, is supplied in similar amounts.

Vitamin D has become one of the most discussed and studied nutrients in human nutrition. This has led to an increased interest in milk and dairy products as sources of vitamin D; and milk is routinely fortified with synthetic vitamin D in many countries. Fortification of milk with synthetic vitamin D is littered with difficulties related to poor stability of naked synthetic vitamin D in watery solutions (Diarrassouba et al. 2015), which can be overcome by securing a naturally high content of vitamin D in the produced milk via the dairy cow (Kurmann & Indyk, 1994).

A sufficient vitamin D status of cattle can be secured by two different vitamin D compounds: vitamin D_2 (D_2) and vitamin D_3 (D_3). Vitamin D_2 originates from roughage, e.g. hay and silage, used for cattle feeding, and it is synthesised during sun-curing at harvest by fungi growing on the plant material (Richardson & Logendra, 1997). Vitamin D_3 is either synthesised endogenously in the skin of the animals during exposure to sunlight (Hymøller & Jensen, 2010b), thus never entering the digestive tract, or supplied in the feed as a synthetic additive. Studies in cattle have shown that D_2 is much less physiologically effective than D_3, and it is not particularly good for securing a sufficient plasma status of the liver derived metabolites 25-hydroxyvitamin D_2 ($25OHD_2$) and 25-hydroxyvitamin D_3 ($25OHD_3$) ($25OHD_x$), respectively (Hymøller & Jensen, 2010a, 2011a). This physiological difference appears illogical from a biochemical point of view since only minor differences in the side-chain of the general steroid ring-structure of the vitamin D molecule exist between D_2 and D_3 (Nigwekar et al. 2012).

In vitamin research there is an on-going discussion about whether synthetic vitamins are as efficient as natural vitamins (Thiel, 2000) and it is often claimed that synthetic vitamins work similar to their natural counterparts in the

*For correspondence; e-mail: Lone.Hymoller@anis.au.dk

organism. However, an increasing number of researchers in physiology, nutrition, and medicine are expressing their doubt, and results in direct opposition to these claims have been published (Thiel, 2000). Many synthetic forms of vitamins are not supplemented in the same physiochemical form as their natural forms but as derivatives stabilised as salts, esters etc. without being chaperoned into circulation by their naturally adjacent cofactors (Thiel, 2000). This is not the case for D_3 because the synthetic dietary form is chemically similar to the natural endogenous form produced in the skin of cattle during exposure to sunlight but the two forms act very different within the living organism, since synthetic dietary D_3 is toxic and can be easily overdosed, in contrast to endogenous natural D_3.

The physiological distinction between natural endogenous and synthetic dietary D_3 in cattle may be facilitated by different transport mechanisms in the blood stream. In a study by Haddad et al. (1993) in humans, natural endogenous D_3 was recovered almost exclusively bound to Vitamin D Binding Protein (VDBP), a protein responsible for storing and transporting vitamin D metabolites in plasma. In contrast, orally administered D_2 was recovered from other plasma fractions than VDBP (Haddad et al. 1993). This has been suggested to be due to dietary D_2 entering circulation through the lymph system after being absorbed with the fat-fraction of the diet in the distal part of the small intestine (Nechama et al. 1977; Maislos et al. 1981; Dueland et al. 1983). The same route of uptake would apply to synthetic dietary D_3. It has, however, never been studied if differences in plasma transport mechanisms between D_2 and D_3 or between natural endogenous and synthetic dietary D_3 is also present in the $25OHD_2$ and $25OHD_3$ metabolites, respectively. Differences in transport mechanisms between vitamin D metabolites in the body of cows could affect the choice of form and route of vitamin D supplementation to secure the highest possible vitamin D content in the produced milk.

The hypotheses of the present study are that differences between routes of plasma transport of $25OHD_x$ exist, which cause the observed physiological inefficiency of D_2 compared to D_3 in securing a sufficient plasma status of $25OHD_x$ in cattle, and cause the difference in toxicity between chemically similar D_3 compounds of endogenous and dietary origin, respectively.

Materials and methods

The study was carried out at Aarhus University, Department of Animal Science in Tjele, Denmark between June 7th and August 16th 2013 at 55°N. The presented study complied with the Danish Ministry of Justice Law No. 1306 (November 23rd 2007) concerning experiments with animals and care of animals used for experimental purposes.

Animals

Six Danish Holstein bull calves were used in the study. The calves were between 9 and 13 d of age at the beginning of the study and weighed between 41 and 56 kg. At the end of the study the calves weighed between 88 and 118 kg. Initial plasma contents of $25OHD_3$ were between 0·3 and 5·7 ng/ml and contents of $25OHD_2$ between 0·2 and 1·3 ng/ml. The coat colour of the calves was assessed from silhouettes of animals (Sneddon et al. 2004) as mainly black or mainly white and the calves were blocked according to predominant coat colour and initial plasma content of $25OHD_3$. Isolated cases of disease among the young calves were treated according to guidelines of the supervising veterinarian.

Treatments, feeding, and housing

The six calves were divided into two treatment groups: Three calves were let out for pasture between 10·00 and 16·00 daily in order to facilitate endogenous D_3 synthesis from sunlight (SUN), whereas the other three calves were housed indoor and fed a synthetic supplement of 75 µg (3·000 IU) D_3 daily (SUP).

Between day 0 and 31 of the study all calves were fed 6 litres of fresh cow milk per day in two servings at 07·00 and 16·30 and in addition the calves in the SUP group were given 75 µg D_3 dissolved in 6 ml ethanol in the milk at the morning feeding. All calves had *ad libitum* access to pelleted concentrate without added synthetic D_3 containing: barley (57%), soybean meal (28%), dried grass pellets (13%), and sugar beet molasses (3%). Between day 32 and day 36 of the study all calves were gradually weaned off milk. Between day 37 and day 71 of the study the calves in the SUN group had *ad libitum* access to the concentrate mixture void of D_3, whereas calves in the SUP group had *ad libitum* access to concentrate (Grønkalv valset, DLG, Copenhagen, Denmark) containing 25 µg (1·000 IU) D_3 per kg. This concentrate consisted of: barley (50%), soybean meal (15%), linseed cake (10%), sugar beet molasses (7%), maize (5%), wheat (4%), and dried grass pellets (3%), palm fat (1%), macro and micro minerals and vitamins (5%). All calves had *ad libitum* access to fresh water and hay at all times. This sun cured hay was the source of D_2 in all calves (Horst et al. 1984).

Before weaning calves were housed in individual pens on straw bedding. After weaning SUN calves were group housed, whereas SUP calves remained individually housed to facilitate registration of individual daily concentrate and, consequently, D_3 intakes. From registered feed intakes it was calculated that calves in the SUP group ate on average 75 µg D_3/d approximately 10 d after weaning, which was equivalent to the amount supplied in the milk prior to weaning.

Sample material

Approximately 30 ml of blood was collected once weekly between 08·00 and 09·00 from the jugular vein in EDTA coated Vacuette ® tubes (Greiner Bio-One GmbH, Kremsmünster, Austria). Blood samples were kept on ice until they were centrifuged for 10 min at 1500 ***g*** (SL 40

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Table 1. Content of triacylglyceride, cholesterol, phosphorlipids, total protein, albumin, vitamin A, and vitamin E in fractions of cattle plasma obtained by gradient ultracentrifugation

	Triacyl-glyceride (mM)	Cholesterol (mM)	Phospho-lipids (mM Choline)	Total protein (g/l)	Albumin (g/l)	Vitamin A† (μg/g)	Vitamin E† (μg/g)
Protein	0·00	1·83	0·06	129	75·3	0·51	0·20
Chylomicron	1·65	2·54	1·54	39·4	26·8	0·29	1·06
LPF1‡	0·50	0·39	0·08	0·00	0·00	0·01	0·11
LPF2‡	0·00	9·75	6·06	34·0	22·4	0·07	3·64
LPF3‡	0·00	2·67	1·88	96·7	71·2	0·51	0·86

†The intake of vitamin A was 9 μg/d and vitamin E was 360 μg/d calculated from registered feed intakes
‡Lipoprotein fractions 1, 2, and 3; LPF1 contains VLDL and LPF2 and LPF3 contain a mixture of LDL and HDL

centrifuge, Thermo Scientific, Hvidovre, Denmark). Plasma was subsequently transferred to micro tubes (Deltalab S.L., Barcelona, Spain) and stored at −18°C until analysis.

For fractioning plasma into five fractions: chylomicron, lipoprotein fraction (LPF) 1, LPF2, LPF3, and protein, 6 ml of plasma was ultra-centrifuged at 100·000 ***g*** for 1 h in a Beckman Coulter 70·1 Ti fixed angle rotor using a Beckman Coulter Optima™ L-80 XP ultracentrifuge (Beckman Coulter Inc., Copenhagen, Denmark). The upper chylomicron fraction was subsequently removed (Axis-Shield, 2011). The remaining plasma was fractionated by gradient ultracentrifugation at 300·000 ***g*** for 18 h after mixing the remaining plasma with 1·5 ml OptiPrep™ (60% iodixanol/water, density 1·32 g/ml) (Axis-Shield, Oslo, Norway) and layering Hepes buffered saline 0·85% (w/v) pH 7·4 on top (Gardner et al. 2003; Axis-Shield, 2011). Fractions of LPF1, LPF2, LPF3, and protein were subsequently removed from the top down by use of Pasteur glass pipettes.

Laboratory analyses

Plasma and plasma fractions were analysed for content of $25OHD_2$ and $25OHD_3$ as described by Hymøller & Jensen (2011b). Proper separation of fractions was confirmed by analysing selected samples for content of triacylglycerides, cholesterol, total protein, and albumin according to standard procedures (Siemens Diagnostics® Clinical Methods for ADVIA 1650) and phosphorlipids according to the Wako Choline Oxidase, DAOS Method. All analyses were performed using an Autoanalyzer ADVIA 1650® Chemistry System (Siemens Medical Solutions, Tarrytown NY, USA). To further confirm the distribution of the obtained fractions, they were also analysed for content of vitamin A and vitamin E as described by Jensen et al. (1998).

Statistics and calculations

Analysis of variance on repeated measures of plasma contents of $25OHD_2$ and $25OHD_3$ was performed using the MIXED models procedure of SAS® (SAS Institute Inc., Cary, NC). The statistical model used was: $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + c_{ijk} + e_{ijk}$ where the dependent variable Y_{ijk} is the content of $25OHD_2$ or $25OHD_3$, μ is the overall mean, α_i is the fixed effect of treatment i (SUN, SUP), β_j is the fixed effect of day j (0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70), $(\alpha\beta)_{ij}$ is the effect of the interaction between treatment i and day j, c_k is the random effect of calf k, and e_{ijk} is the random residual error. To account for the covariance structure of the repeated measures during consecutive days within calf the covariance was modelled using the repeated statement in the MIXED procedure of SAS® (Littell et al. 2006). The best model fit was obtained using the auto regressive first order covariance structure. Random effects were assumed normally distributed with mean value zero and constant variance $c_{ijk} \sim N\ (0,\ \sigma_c^2)$ and $e_{ijk} \sim N\ (0,\ \sigma^2)$. Distribution of $25OHD_2$ and $25OHD_3$ between different plasma fractions in each sample was calculated as the amount (ng) of a given metabolite found in each fraction in per cent of the total amount (ng) found in all fractions. Differences were compared by Student's two-sample, two-tailed heteroscedastic t-test on averages of all samples between calves and sampling times. Differences were considered statistically significant when $P \leqslant 0{\cdot}05$ and as tendencies if $0{\cdot}05 < P \leqslant 0{\cdot}1$.

Results

Contents of lipids, proteins, vitamin A, and vitamin E in the different plasma fractions are shown in Table 1. Total protein was highest in the protein fraction whereas no protein or albumin was encountered in LPF1. Cholesterol and phospholipids were mainly found in LPF2 and LPF3 whereas triacylglyceride was mainly found in chylomicrons. The highest concentration of vitamin A was encountered in the protein fraction and the highest concentration of vitamin E in the LPF2 fraction.

Plasma contents of $25OHD_3$ were not affected by treatment ($P = 0{\cdot}36$) but there was a significant effect of day ($P \leqslant 0{\cdot}001$) and a significant interaction between treatment and day ($P \leqslant 0{\cdot}05$), probably due to the plasma content of $25OHD_3$ increasing more rapidly in SUN than in SUP during the study. However, the only differences between plasma contents of $25OHD_3$ in SUN and SUP were found at day 28 ($P = 0{\cdot}08$) and day 35 ($P = 0{\cdot}05$), where SUN had a higher plasma content of $25OHD_3$ than SUP.

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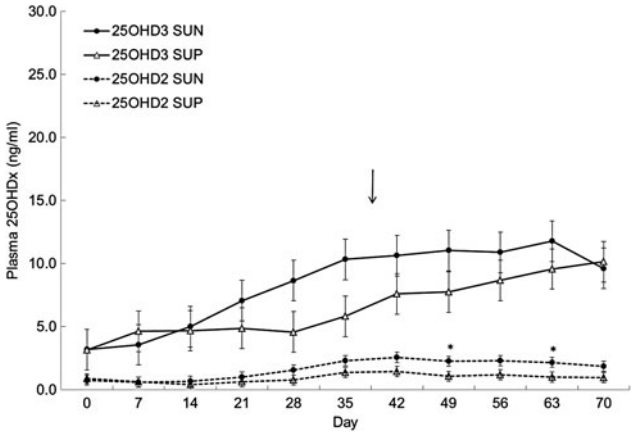

Fig. 1. Plasma concentration (mean ± SEM) of 25(OH)-vitamin D_3 ($25OHD_3$) and 25(OH)-vitamin D_2 ($25OHD_2$) in calves supplied with vitamin D_3 from the sun (SUN) or from synthetic supplements (SUP) during 70 d ($n = 3$). Arrow indicates time of weaning. *Significant difference between SUN and SUP in plasma content of $25OHD_2$ or $25OHD_3$ *$P \leqslant 0{\cdot}05$, **$P \leqslant 0{\cdot}01$, ***$P \leqslant 0{\cdot}001$.

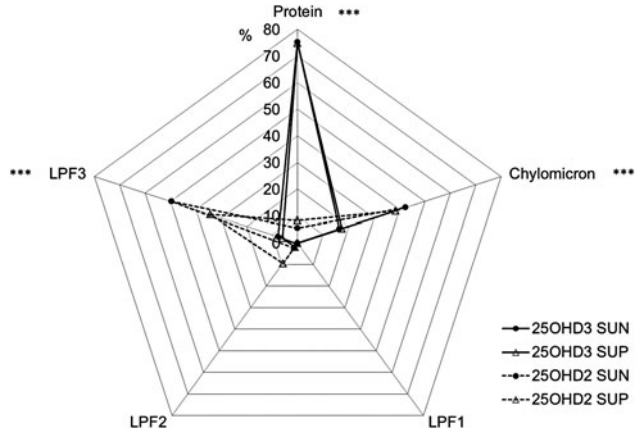

Fig. 2. Distribution (% of total content in all fractions) of 25(OH)-vitamin D_3 ($25OHD_3$) and 25(OH)-vitamin D_2 ($25OHD_2$) in plasma fractions of protein, chylomicrons, and lipoprotein fractions (LPF) in calves supplied with vitamin D_3 from the sun (SUN) or from synthetic supplements (SUP) during 70 d (mean of all samples, $n = 3$). LPF1 equals VLDL and LPF2 and LPF3 is a mixture of LDL and HDL. *Significant difference between fraction content of $25OHD_2$ and $25OHD_3$ in both SUN and SUP *$P \leqslant 0{\cdot}05$, **$P \leqslant 0{\cdot}01$, ***$P \leqslant 0{\cdot}001$.

Plasma contents of $25OHD_2$ were generally very low and were, as expected due to its source in the hay fed to all calves, not affected by treatment ($P = 0{\cdot}14$) but there was a significant effect of day ($P \leqslant 0{\cdot}001$). No interaction between treatment and day was found ($P = 0{\cdot}76$) but plasma contents of $25OHD_2$ were higher in SUN than in SUP between day 42 and 63 ($P \leqslant 0{\cdot}06$) (Fig. 1).

The percentage of $25OHD_3$ was higher than the percentage of $25OHD_2$ in the protein fraction ($P \leqslant 0{\cdot}001$), whereas the percentage of $25OHD_2$ was higher than the percentage of $25OHD_3$ in the chylomicron ($P \leqslant 0{\cdot}001$), the LPF2 ($P \leqslant 0{\cdot}05$), and the LPF3 ($P \leqslant 0{\cdot}001$) fractions. No $25OHD_2$ or $25OHD_3$ was found in the LPF1 fraction (Fig. 2).

Discussion

Separation between fractions of protein, chylomicrons, and LPF1, which contains very low density lipoprotein (VLDL), was obtained and confirmed by their individual contents of proteins, lipids, and fat soluble vitamins. Based on the contents of proteins, lipids, and fat soluble vitamins, the fractions LPF2 and LPF3 appeared to contain a mixture of low density lipoprotein (LDL) and high density lipoprotein (HDL); whereas the chylomicron fraction appeared to contain traces of VLDL. Particularly LDL and HDL can be difficult to separate completely in cattle plasma, when using methods developed for human plasma fractionation (Gardner et al. 2003), and overlaps in density between LDL and HDL in cattle, particularly in calves, are previously reported (Bauchart, 1993; Leplaix-Charlat et al. 1996). Furthermore, in humans LDL is the main cholesterol transporter (Kayden & Traber, 1993), whereas in cattle a significant proportion of cholesterol is found circulating in HDL (Herdt & Smith, 1996; Leplaix-Charlat et al. 1996; Pysera & Opalka, 2000). This may further contribute to an ineffective separation of cattle LDL and HDL in density gradients developed for human plasma. In the present study it appeared, from the contents of cholesterol and vitamin E in the LPF2 and LPF3 fractions, that they contained a mixture of LDL and HDL. Cholesterol contents were slightly higher in LPF2 than LPF3 and the highest vitamin E concentration was found in LPF2. Vitamin E is reported by others to be circulating in the highest concentration in HDL in cattle or at least in even concentrations between LDL and HDL in ruminants (Herdt & Smith, 1996; Senaidy, 1996; Ametaj et al. 2000). Hence it appears that satisfactory separation of plasma fractions was obtained.

Regardless of origin, endogenous D_3 or synthetic D_3, respectively, $25OHD_3$ was similarly distributed between plasma fractions, which indicated that differences in toxicity between endogenous and synthetic D_3 must be found at other levels of the vitamin D metabolic pathways than in the 25-hydroxylated metabolites. All calves had the same plasma content of $25OHD_2$ and $25OHD_3$, respectively, regardless of the treatments and there was no difference between treatments in distribution of $25OHD_2$ and $25OHD_3$, respectively, in different plasma fractions. However, when comparing the distribution of $25OHD_2$ to the distribution of $25OHD_3$, it became obvious that the two forms of hydroxylated vitamin D circulate bound to completely different plasma fractions. Haddad et al. (1993) found in humans that endogenously synthesised D_3 is recovered almost exclusively bound to VDBP whereas D_2 supplemented orally is recovered from other plasma fractions e.g. chylomicrons. This difference is speculated to be

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caused by dietary D_2 entering systemic circulation through the lymph system after absorption with the fat fraction of the feed (Nechama et al. 1977; Maislos et al. 1981; Dueland et al. 1983). However, results from the present study showed that this difference in distribution between different plasma fractions is also found between $25OHD_2$ and $25OHD_3$, which can be caused by a low binding affinity of $25OHD_2$ to VDBP. The protein fraction, which should cover most of the circulating VDBP designated to binding and transporting vitamin D, contained almost 80% of the circulating $25OHD_3$ whereas next to none of the circulating $25OHD_2$ was encountered in this fraction. In contrast $25OHD_2$ was found in chylomicron and LPF3 fractions, which may not offer the same level of protection to the molecule against degradation or excretion as binding to designated transport proteins. This could be part of the explanation to why D_2 is less efficient at securing a sufficient vitamin D status in cattle, measured as $25OHD_2$, than D_3, measured as $25OHD_3$, as observed by Hymøller & Jensen (2010a, 2011a). However, the consequences of the difference in the plasma transport of different vitamin D metabolites for their transfer into milk needs investigation.

Conclusions

Transport of $25OHD_2$ and $25OHD_3$ in different plasma fractions may explain the relatively low physiological efficiency of D_2 compared to dietary D_3 in cattle. However, no difference in plasma transport between $25OHD_3$ of synthetic dietary or natural endogenous origin exist, hence the physiological distinction and discrimination between these two D_3 sources must be related to other levels of the vitamin D metabolic pathway than the liver derived $25OHD_3$.

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