The Role of Vitamin D on Calcium Metabolism in Horses

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Summary

Concentrations of $25OHD_3$ and $1,25\text{-}(OH)_2D_3$ in plasma of horses are much lower ($25\ OHD_3 < 5.0$ nmol/l) than in plasma of most other domestic animal species. From this, experiments were carried out to examine the physiological importance of vitamin D in regulating calcium metabolism in horses. 1) Intramuscular administration of 26 µmoles/100 kg bw. of vitamin D_3 raised the vitamin D_3 concentration in plasma from 5.2 up to 729 nmol/l but induced no increase in the concentration of $25OHD_3$ and $1,25\text{-}(OH)_2D_3$. 2) The concentration of ionic calcium in digesta of the small and large intestine (0.2 to 1.2 mmol/l) was significantly lower than in blood plasma (1.7 mmol/l) and contrasted with that of $[Mg^{2+}]_i$. This indicated active intestinal absorption of calcium. 3) The in vitro activity of the renal cortex lα-hydroxylase was undetectable, except for one animal. It appears that the influence of the vitamin D system on calcium metabolism, if any, is not as great in horses under normal conditions as it is in other domestic animals.

Die Rolle von Vitamin D für den Calciumstoffwechsel bei Pferden

Im Blut von Pferden ist die Konzentration von $25OHD_3$ (Calcidiol) und $1,25\text{-}(OH)_2D_3$ (Calcitriol) mit < 5,0 nmol/l (Calcidiol) wesentlich niedriger als im Plasma vieler anderer Haussäugetiere. Es wurden daher Versuche durchgeführt, um die physiologische Bedeutung von Vitamin D für die Regulation des Calciumstoffwechsels beim Pferd zu untersuchen. 1) Eine intramuskuläre Injektion von 26 µmol Vitamin D_3/100 kg KM erhöhte die Vitamin-D_3-Konzentration im Plasma von 5,2 auf 729 nmol/l. Dies führte jedoch zu keinem Anstieg von $25OHD_3$ und $1,25\text{-}(OH)_2D_3$. 2) Die Konzentration des ionisierten Calciums in der Ingesta des Dünn- und Dickdarms (0,2 bis 1,2 mmol/l) war, im Gegensatz zum ionisierten Magnesium, signifikant niedriger als im Plasma (1,7 mmol/l). Dies deutet auf einen aktiven intestinalen Calciumtransport im Darm hin. 3) Die Aktivität der lα-Hydroxylase der Nierenrinde war, außer bei einem Tier, nicht meßbar. Die Versuche deuten darauf hin, daß das Vitamin-D-System bei Pferden unter normalen Bedingungen, wenn überhaupt, eine geringere Bedeutung hat als bei anderen Haustieren.

Introduction

The concentration of 25-hydroxyvitamin D (25OHD) in plasma is regarded a reliable index of vitamin D-status in human and animals (*Mawer* et *al.*, 1985). The concentration of this metabolite in plasma of normal adults and most domestic animal species ranges between 50 and 112 nmol/l (20 and 45 ng/ml) (*Horst* et *al.*, 1981; *Bikle* et *al.*, 1986), with little differences between adult and growing individuals (*Horst* and *Littledike*, 1982). Somewhat higher values of $25OHD_3$ are normally found in summer and fall (*Mäenpää* et *al.*, 1988 b, *Mäenpää* and *Lappeteläinen*, 1987). For both, humans and animals, $25OHD_3$ concentrations in plasma below 25 to 38 nmol/l (10 to 15 ng/ml) are considered as suboptimal.

In contrast to this, $25OHD_3$ concentrations in plasma of horses have been reported to be usually much lower with values below 5.0 nmol/l (*Mäenpää* et *al.*, 1988 a, *Smith* and *Wright*, 1984). There was also not much of seasonal increase in the plasma $25OHD_3$ concentration in horses during the summer period (*Mäenpää* et *al.*, 1988 b), with the concentration raising from 5.8 in January to only 7.3 nmol/l in October.

Such low levels of $25OHD_3$ could either result from ineffective cutaneous production of vitamin D_3 or from low $25OHD_3$ synthesis in the liver. In addition, it raises the question about the physiological importance of vitamin D in regulating calcium metabolism in horses.

In order to examine the responsiveness of the vitamin D metabolic system, pharmacological amounts of vitamin D_3 were intramuscularly administered to horses and the concentrations of various vitamin D_3 metabolites in plasma were measured.

In another series of experiments the concentration of ionized calcium, $[Ca^{2+}]_i$, in chyme from various segments of the small and large intestines were determined, to evaluate the site of its absorption in the gut and to get hints about its mechanism of absorption.

Furthermore, it has been reported that both, calcium concentration in plasma and calcium balance, increased in horses when the animals are fed a pure lucerne hay diet compared to feeding a pure concentrate diet (*Meyer* et *al.*, 1990). In order to see, whether those dietary factors might affect the calcium status, independent from vitamin D, or might influence the renal pattern of vitamin D metabolism. The renal lα-hydroxylase and 24-hydroxylase were measured in equine renal cortex preparations from animals fed either a pure grass hay or a concentrate-rich diet.

Material and Methods

Experimental design for application of pharmacological amounts of vitamin D_3.

Three pony mares of about 180 kg body weight with an age of nine to ten years were fed ad lib. for three weeks a hay/oat ration. The food was offered twice daily. The intake of calcium and phosphate was 3.31 g and 3.00 g/(100 kg bodyweight.d), respectively. A single dose of 26 µmoles (10 mg) of vitamin D_3 per 100 kg body weight, in water soluble form, was intramuscularly inject-

ed at 7.30 h, before the morning feed. Twenty-three blood samples, of 20 ml each, were collected from two days before until seven days after vitamin D_3 administration. The concentrations of calcium and the activity of the alkaline phosphatase in plasma were determined. In addition, concentrations of vitamin D_3, $25OHD_3$, $24,25\text{-}(OH)_2D_3$ and $1,25\text{-}(OH)_2D_3$ were measured in selected samples of plasma following the method of *Kaune* and *Harmeyer* [1986].

Sampling and measurement of ionized calcium in chyme.
Eight ponies (4 males and 4 females) between 6 and 18 years old with 150 to 240 kg body weight were either fed a grass hay diet (four animals) or a corn/wheatbran diet (four animals) for eight weeks. The daily intakes of calcium were 10.2 g/100 kg bodyweight for the hay diet and 11.2 g for the corn/wheatbran diet. The corresponding values of intake of phosphorus were 5.9 and 10 g and for vitamin D 3.2 and 325 nmoles/(100 kg.d), respectively. The animals were killed five hours after the morning feed at 10 h a. m. by stunning and bleeding. Total digesta was collected in separate portions from the stomach, duodenum and ileum, from the caecum, the ventral and dorsal colon, and from the descending colon. This was achieved within about 30 min after bleeding. Representative subsamples of ingesta were taken from the different intestinal segments and were centrifuged at $3 \cdot 10^3$ g (ingesta from small intestine) and at $15 \cdot 10^3$ g (ingesta from large intestine) for 15 min. The pH-values and concentrations of ionized calcium and ionized magnesium were measured in the supernatants and in six blood samples from each animal which were collected from the horses at weekly intervals before sacrificing the animals.

Sample preparation for measurement of renal vitamin D metabolism.
The left kidney was removed from the abdominal cavity about three minutes after bleeding the animal. A branch of the renal artery was perfused with 150 ml of icecold tris-acetate buffer of pH 7.4 with 1 mmol/l EDTA and 200 mmol/l saccharose to remove plasma residues from the tissue. After removal of the serosa, renal cortex from the perfused area was sliced off and cut with scissors into about 2.2 mm pieces. The suspended pieces of tissue were washed three times with icecold tris-acetate buffer of pH 7.4 and separated from the solution by rinsing through a screen. Three grams of tissue was resuspended in tris-acetate/succinate buffer with 1.9 mmol/l $MgCl_2$ and 200 mmol/l saccharose. The suspension was homogenized two times for 3 sec with a blender (Fa. Janke & Kunkel KG, Staufen, FRG) and subsequently with 3 strokes of a Potter-Elvejhem homogenizer. A 5 percent homogenate was centrifuged at 600 g for 10 min. at 0°C. Three ml of supernatant were gassed with oxygen, preincubated for 5 min at 37°C. A constant amount of [26-^{3}H]-methyl-$25OHD_3$ was added with increasing substrate concentrations of 26; 102; 297; $1.04 \cdot 10^3$ and $2.08 \cdot 10^3$ nmol/l and the mixtures were incubated for 10 min at 37°C in the presence of 100 percent oxygen. The reaction was stopped by adding 6 ml of methanol. Labeled $25OHD_3$ and newly formed vitamin D-metabolites were extracted from the incubation mixture and quantified after HPLC-separation (*Schreiner* and *Harmeyer*, 1986).

Laboratory methods.
Quantification of calcium and activity of alkaline phosphatase was achieved by using standard methods. Ionized calcium and ionized magnesium were potentiometrically measured with ionselective electrodes[1].
Vitamin D and vitamin D-metabolites in plasma were quantitatively determined in methanol/methylene chloride extracts (2 + 1). The extracts were fractionated on columns filled with Lipidex 5000 (Fa. Packard Instruments) using an n-hexane/chloroform gradient. The vitamin D metabolites were separated under normal-phase conditions as described earlier (*Kaune* and *Harmeyer*, 1986). In kidney homogenates vitamin D-metabolites were extracted with chloroform, filtered, dried, and dissolved in isopropanol/n-hexane for HPLC separation. Calcitriol ($1,25\text{-}(OH)_2D_3$) was quantified after HPLC separation with an $1,25\text{-}(OH)_2D_3$ antibyody from sheep (*Kaune* and *Harmeyer*, 1986). UV-absorbance and radioactivity of $25OHD_3$ and other labeled vitamin D-metabolites were simultaneously recorded after HPLC chromatography with a photodiode array detector (Fa. Waters, Eschborn, FRG) and a radioactivity monitor LB 507A (Fa. Berthold GmbH & Co., Wildbad, FRG). The size of the flow cell was 1.0 ml.

[1] The Mg^{2+}-sensitive electrode was kindly provided for this study by Fa. AVL Gesellschaft für Medizinische Meßtechnik mbH, Bad Homburg

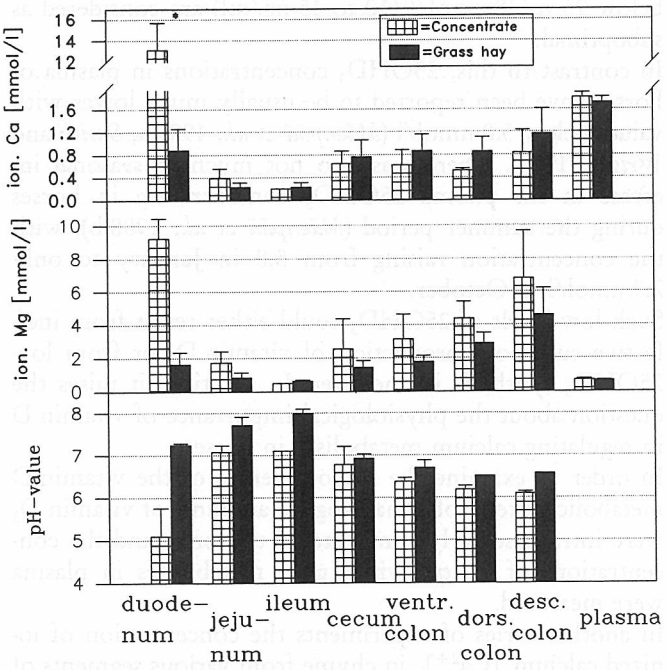

Fig. 1: $[Ca^{2+}]_i$ (top), $[Mg^{2+}]_i$ (middle) and pH values (bottom) of ingesta from various segments of small and large intestine and plasma ($\bar{x} \pm SD$, n = 3 per group); * measured after dilution 1 + 4 with deion. water yielding too high values.

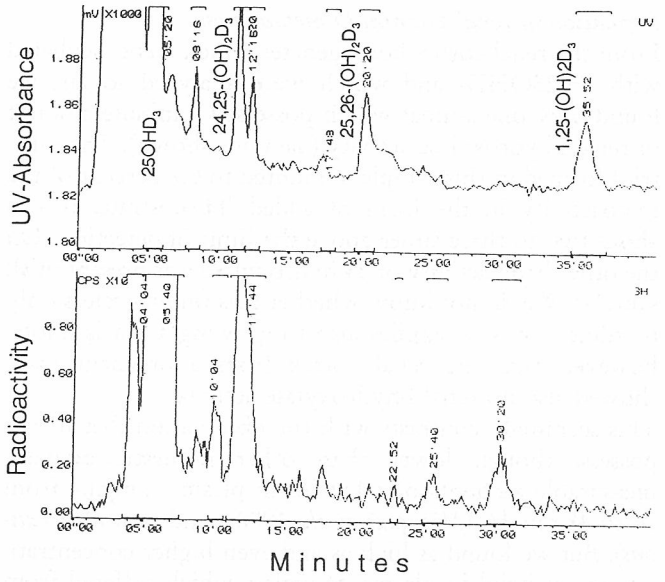

Fig. 2: UV-chromatogram and radiochromatogram, simultaneously recorded from an extract of renal cortex homogenate incubated for 10 min with $2.08 \cdot 10^3$ nmol/l ^{3}H-25OHD$_3$. Besides labeled 24,25-$(OH)_2D_3$ there are two unidentified radioactive peaks at retention times of 25.4 and 30.2 min. No radioactivity is visible at the position of 1,25-$(OH)_2D_3$. Unlabeled 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ were added to the extract after incubation in order to locate the position of these substances in the UV-chromatogram.

Results

Injection of pharmacological doses of vitamin D_3.
The concentrations of calcium (2.94 ± 0.17 vs. 2.99 ± 0.18 mmol/l), and the activity of alkaline phosphatase (349 ± 70 vs. 356 ± 53 U/l) in plasma were not influenced by a single intramuscular injection of 26 µmoles of vitamin D_3/100 kg bodyweight. The concentration of vitamin D_3 in plasma increased after the injection from < 5.2 nmol/l to 390 and 729 nmol/l and peaked about 2 d post injection. The concentration remained significantly elevated (> 130 nmol/l) at least until 6 d post injection.
The concentration of 25OHD$_3$ in plasma was about 10 nmol/l before the vitamin D_3 administration and remained unchanged by administration of vitamin D_3. The same was true for 24,25-$(OH)_2D_3$ whose concentration was between 7.2 to 12.0 nmol/l. Concentration of calcitriol in plasma was between 36 to 96 pmol/l but showed also no increase due to the administration of vitamin D_3.

Concentration of ionized Ca^{2+} in chyme of hay or concentrate fed horses.
Except for the duodenum from concentrate fed horses, the concentration of ionized calcium, corrected to pH 7.4, was lower in all segments of the small and large intestine compared to blood plasma (about 1.7 mmol/l) and constituted a concentration difference between these compartments of the body (Fig. 1). The $[Ca^{2+}]_i$ in jejunal and ileal chyme was between 0.2 and 0.4 mmol/l exhibiting a ratio of about 1 : 6 compared to blood plasma. In the hindgut of grass hay fed animals $[Ca^{2+}]_i$ was higher than in animals receiving concentrate. This difference was associated with a slight but significantly lower $[Ca^{2+}]_i$ concentration in plasma (1.71 ± 0.09 vs. 1.83 ± 0.06 mmol/l; $\bar{x}$ ± SD; n = 30 per group) in the grass hay fed horses as compared to the concentrate fed animals. The concentration of ionized magnesium in the intestine was always higher than that present in blood plasma (0.66 ± 0.054 mmol/l) (Fig. 1). In contrast to $[Ca^{2+}]_i$, $[Mg^{2+}]_i$ in the large intestine was always higher in horses which received concentrate diet compared to feeding hay. This indicated that, irrespective of changes in chyme volume, either relatively more calcium than magnesium disappeared from, or less calcium than magnesium entered into the hindgut segments. The pH-values in chyme of different intestinal segments were generally lower in horses which received concentrate compared to feeding hay.

Formation of 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ by renal cortex homogenates.
Five percent renal cortex homogenates were incubated with tritium labeled 25OHD$_3$ for ten min. The amount of radioactivity, converted into 24,25-$(OH)_2D_3$, decreased from 18 to 1.5 percent of that present in 25OHD$_3$ when the substrate concentration increased from 26 to $2.08 \cdot 10^3$ nmol/l (Fig. 2). If any 1,25-$(OH)_2D_3$ was formed under these conditions it was, except for one animal, below the limit of detection (Fig. 2). This demonstrated that the activity of the renal cholecalciferol-l-hydroxylase is very low in adult horses. Fig. 2 shows an UV-chromatogram and a radiochromatogram obtained from a renal cortex homogenate preparation from a horse which was pregnant for six months. From other domestic animal species it is known that the renal l-hydroxylase activity increases during pregnancy. The renal cortex was incubated with $2.08 \cdot 10^3$ nmol/l 25OHD$_3$ for 10 min.

Discussion

Application of pharmacological amounts of vitamin D.
Intramuscular administration of 26 µmoles of vitamin D_3/100 kg bodyweight raised the vitamin D_3 concentration in plasma about hundredfold but had no detectable effect on 25OHD$_3$ concentrations in plasma. *Harrington* and *Page* (1983) offered daily 22 µmoles of vitamin D_3/100 kg bodyweight to a male horse and found an increase in 25OHD$_3$ concentration in plasma from an undetectable low level up to 502 nmol/l. It appears that a single dose of 26 µmol of vitamin D_3/100 kg was too low to affect vitamin D-metabolism in horses. From this, it is not surprising that the plasma calcitriol concentration was also unaffected by the vitamin D administration. In humans and many domestic animal species, production of calcitriol is more tightly controlled than that of 25OHD$_3$ (*Napoli* and *Horst*, 1984). In the horse fed with 22 µmoles of vitamin D_3/100 kg bodyweight daily by *Harrington* and *Page* (1983), calcitriol concentration in plasma (61 pmol/l) remained unchanged over 4 weeks although the animal exhibited severe symptoms of vitamin D intoxication after about one week of feeding vitamin D_3. It appears that the extra vitamin D which was administered in our experiment

was largely eliminated by other probably unconventional, metabolic pathways. It also indicates that horses possess only a comparably low hepatic 25-hydroxylase or exert considerable control upon this enzyme. This perhaps differs from that observed in other domestic animals. When a dose of vitamin D_3 comparable to that administered to the horses, is intramuscularly administered to 20 kg piglets the plasma $25OHD_3$ concentration raises about 2 to 3 fold (from 100 to 250 nmol/l) (own observation). A similar dose injected into non-lactating, non-pregnant bovines leads to a comparable increase in vitamin D_3 in plasma as in horses and pigs. But $25OHD_3$ in plasma increases less than in pigs and decays more slowly than in the pig (*Napoli* and *Horst*, 1984).

Concentration of ionized calcium in digesta. The lower $[Ca^{2+}]_i$ concentration in the chyme in all segments of the intestine as compared to plasma strongly indicate that calcium in the horse, like in other domestic animal species, is actively absorbed. It appears unlikely that the concentration difference of $[Ca^{2+}]_i$ results from solvent drag or any other non-active mechanism, possibly associated with net water outflow from the intestine. In this case, one would expect a similar effect on $[Mg^{2+}]_i$, which, however, was not observed. Surely, $[Mg^{2+}]_i$ is not an intestinal marker. The daily intake of magnesium, however, was less than 10 percent that of calcium and $[Mg^{2+}]_i$ in jejunum was about five times the $[Ca^{2+}]_i$ concentration. At least at present it appears unlikely that secretion of Mg into the intestine can entirely account for this difference. From reports by others it is also likely, that intestinal calcium absorption in horses responds to vitamin D. Those studies have shown that calcium balance increased (*Hintz* et *al.*, 1973) and that the plasma calcium concentration was raised significantly after oral administration of toxic amounts of vitamin D (*Muylle* et *al.*, 1974, *Harrington*, 1982, *Harrington* and *Page*, 1983), or after ingestion of a shrub (Cestrum diurnum), which contains vitamin D-like activity (*Krook* et *al.*, 1975). Conversely, lactating mares, probably with low vitamin D-status, may develop hypocalcemia and eclampsia (*Rach* et *al.*, 1972, *Meijer*, 1982, *Richardson* et *al.*, 1991). But it is unknown whether the abnormality can be prevented by the presence of physiological amounts of vitamin D. Whether vitamin D plays a significant role in regulating calcium metabolism under normal conditions remains still unanswered.

The positive effect of a lucerne diet on plasma calcium concentration reported in a previous study (*Meyer* et *al.*, 1990) could not be demonstrated in these experiments where the horses were fed grass hay instead of lucerne hay but were offered only about one-third the amount of calcium (10.2 vs. 27.8 g/(100 kg.d). It is, however, of interest to note, that the slightly lower concentration of calcium in plasma in the hay fed animals was associated with a higher $[Ca^{2+}]_i$ in the large intestine compared to horses which received concentrate.

Formation of renal vitamin D metabolites.
From the renal cortex homogenates which were incubated with ^3H-$25OHD_3$ and which were analyzed so far, we found only one animal which possessed a minute amount of renal l-hydroxylase activity (data not shown). The calcitriol formed in this sample amounted to 0.7 percent of the radioactivity in the form of added ^3H-substrate. It was about two to three times above the limit of detection. On the other hand activity of 24-hydroxylase was present in all samples. We do not know whether this finding refers only to adult horses or applies also to growing animals. Note, however, that the renal cortex from a pregnant mare showed also no renal l-hydroxylase activity.
This seemingly contrasts with the observation that horses possess, though lower than other domestic animals, measurable amounts of calcitriol in plasma, ranging from 36 to 48 pmol/l (*Mäenpää* et *al.*, 1988 a and *own observations*). But we found as high as and even higher concentrations of calcitriol in plasma of piglets which suffered from pseudovitamin D-deficiency rickets, type I (*Kaune* and *Harmeyer*, 1987). These animals lack the normal l-hydroxylase, due to a genetic defect and inevitably develop rickets at the age of weaning. Calcitriol concentrations in plasma, comparable to those or even higher than those found in horses, are also present in anephric piglets (*Horst* et *al.*, 1981, *Littledike* and *Horst*, 1982) and anephric humans (*Dusso* et *al.*, 1988). Thus, at present one cannot exclude that at least some of the calcitriol circulating in horse plasma is of extrarenal origin.

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