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Undetectable vitamin D_3 in equine skin irradiated with ultraviolet light

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Vitamin D requirements for most animals are expected to be fulfilled through daily exposure of the skin to solar ultraviolet B radiation. The synthesis of vitamin D_3 in skin depends on different factors including melanin pigmentation, the amount of UVB radiation reaching the skin, type of clothing/hair coat, latitude and altitude, season, and time of day. Alternatively vitamin D_2 may be obtained from UVB irradiated pasture species. Recent studies have shown that in unsupplemented grazing horses 25-hydroxyvitamin D_2 is the predominant form of vitamin D in plasma, and that 25OHD_3 is undetectable suggesting horses may rely on diet to obtain vitamin D. In order to mimic the natural environment of skin to sunlight exposure, five equine and two ovine devitalized skin samples were irradiated with 5 J/cm^2 of UVB light followed by measurement of 7-dehydrocholesterol (7-DHC) and vitamin D_3 concentrations using reverse-phase high pressure liquid chromatography (HPLC). HPLC revealed the presence of 7-DHC in the skin of both horses and sheep. Vitamin D_3 was undetectable in both ovine and equine skin prior to irradiation, but after irradiation with UVB light, ovine skin showed an increase in vitamin D_3 concentration (mean 0.16 ± 0.07 µg/g), whereas vitamin D_3 was undetectable in equine skin. These results provide additional evidence that horses make negligible quantities of vitamin D_3 in their skin after exposure to UVB light and may therefore rely on their diet as a primary source of vitamin D.

Key words: *7-dehydrocholesterol, cholecalciferol, horse, skin, ultraviolet light*

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Vitamin D is known to play an essential role in the maintenance of calcium homeostasis and skeletal metabolism. In some animal species, the physiological requirement for vitamin D is mostly fulfilled by the conversion of 7-dehydrocholesterol (7-DHC) in the skin, through the influence of solar ultraviolet B radiation (UVB) (approximately 290–325 nm), to previtamin D_3, followed by thermal isomerisation at 37°C to vitamin D_3 [5, 10]. Other sources of vitamin D include: consumption from natural sources such as fatty fish (vitamin D_3), and irradiated plants (vitamin D_2) or from supplemented foods (vitamin D_3) [6, 19]. Vitamin D enters the circulation where it binds to vitamin D-binding protein (DBP) and is transported to the liver where it undergoes hydroxylation to 25-hydroxyvitamin D and then to the kidney where a second hydroxylation occurs forming the active form of vitamin D, 1,25-dihydroxyvitamin D [5, 6, 9].

The photoconversion of 7-DHC to vitamin D_3 has a direct correlation with the amount of ultraviolet B irradiation that reaches the skin [4]. In humans, the sum of exposure to ultraviolet B photons plus the dietary intake are considered important contributions to vitamin D status. These can be influenced by lifestyle, culture, traditional diets, behavioural parameters, geographic location (latitude and altitude), season, time of day, cloud cover/pollution, amount of sun exposure, melanin quantity in the skin, age, the degree of protection from sunlight, and clothing traditions [4, 10].

Studies in herbivores have shown that sheep [3, 14], goats [17, 18] and cows [12] are able to produce vitamin D_3 in their skin. A study looking at the natural history of vitamin D metabolism in New Zealand native species found that kiwi (*Apteryx mantelli*), tuatara (*Sphenodon punctatus*) and New Zealand sea lions (*Phocarctos hookeri*) all produced vitamin D_3 in the skin [15]. Kiwi are a nocturnal bird, and contrary to expectations a small amount of vitamin D_3 production did occur in irradiated skin, meaning despite evolution selection pressures kiwi had not lost the ability for dermal vitamin

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D_3 production [15]. In contrast, experimental studies on carnivores, mainly cats and dogs, have demonstrated they do not synthesise vitamin D_3 in their skin and rely on their nutrition and dietary supplements to fulfil their vitamin D needs [11, 20].

In previous studies we showed that 25-hydroxyvitamin D_2 was the predominant form of vitamin D in equine serum and that 25-hydroxyvitamin D_3 was either very low or below detection limits of the assay [1, 2]. Therefore, the main aim of this study was to investigate whether horses, like other herbivores, are able to synthesise vitamin D_3 in their skin in an experimental setting.

Materials and Methods

Skin samples were collected post-mortem from animals undergoing euthanasia (Pathobiology Section post-mortem room, Massey University, Palmerston North) for reasons unrelated to the experiment. Skin samples were excised within 30 min of euthanasia from the forehead, dorsum, and legs of five Thoroughbred gelding horses (mean age of 5 ± 1.4 years), three brown and two bay in colour, and the dorsum of two adult Romney cross female sheep. The skin samples were placed into sterile plastic storage tubes, wrapped in aluminium foil and stored at −80°C until analysis.

Prior to irradiation, the skin was thawed, and hair and subcutaneous tissue were removed in a dark room. Skin samples from each animal were pooled, measured and divided equally into two different groups, each 10 cm^2 in size. One skin pool from each animal was not irradiated and just wrapped in the aluminium foil to be used as a control sample. The other skin pool from each animal was irradiated with an ultraviolet lamp (UV-Lampe für Reptilien und Vögel, Lucky Reptile, Waldkirch GmbH, Germany) with output of between 290–305 nm. Each pool was irradiated for 8 hr to obtain at least 5 J/cm^2 of accumulated UVB [8, 11, 23]. The ultraviolet lamp was used with an optimised reflector box (size: width 28 cm^2, length 48 cm^2, depth 24 cm^2) and UVB light was measured in μW/cm^2 by Solarmeter Model 6.2 UVB meter (Solartech Inc., Harrison Township, MI, USA) in the 280–320 nm wavelength.

During irradiation, all samples were kept in a 37°C room to allow for thermal isomerisation [23], and were moistened regularly with phosphate buffered saline. After irradiation, all samples were cut into small pieces (5 × 5 mm) in a dark room. The skin samples were weighed and freeze-dried using a freeze drier (Cuddon Freeze Dry model 0610, Blenheim, New Zealand) prior to saponification and lipid extraction.

The skin samples were processed as previously described [13, 15, 20] and outlined in full below. The skin pools underwent ethanolic saponification with potassium hydroxide (KOH) as follows: the samples were combined with 0.1 ml of sodium ascorbate (0.2 g/ml), 8 ml of 1% ethanolic pyrogallol (1 g pyrogallol/100 ml ethanol) (Absolute Ethanol (200 proof), Molecular Biology Grade, Fisher BioReagents™, ThermoFisher Scientific, MA, USA, pyrogallol MP Biomedicals™, ThermoFisher Scientific) and 60% KOH (Analytical Reagent Grade, ThermoFisher Scientific) in water in a round bottom flask with a stirring bar and refluxed over a hot plate stirrer. After cooling, the digest was filtred in a Buchner funnel with filter paper under partial vacuum. To perform lipid extraction, the residue on the filter paper was washed with ethyl acetate (HPLC Grade, Fisher Chemical, ThermoFisher Scientific) (80:20) and then n-hexane (HPLC Grade, Fisher Chemical, ThermoFisher Scientific), the combined filtrate was then transferred to a separating funnel, and water was added. The filtrate was extracted two more times with hexane and the combined hexane extracts were washed three or more times with water. Using a rotor vapor (Rotavapor® B-3, BÜCHI, Labortechnik AG., Flawil, Switzerland) at 38 ± 2°C, the samples were evaporated and the subsequent residue reconstituted with HPLC grade methanol (HPLC grade, Fisher Chemical, ThermoFisher Scientific), followed by sample clean-up using solid phase cartridges SPE C18 (EC 55 μm, 70A, Phenomenex, CA, USA) in order to remove subcutaneous fat. The cartridge was activated with methanol (Fisher Chemical, ThermoFisher Scientific) and the sample washed with 70% methanol, followed by elution with the mobile phase (95% methanol in water), which was collected and dried under nitrogen.

Reverse phase high-performance liquid chromatography (HPLC) was performed using a Luna C-18 column (250 × 46 mm ID, 5 μm particle size) (Phenomenex), with an isocratic mobile phase of methanol: H_2O (95:5), at a flow rate of 1.2 ml/min. Vitamin D_3 and 7-DHC were detected using an ultraviolet detector at 265 nm. Controls included vitamin D_3 (Sigma-Aldrich Corp., St. Louis, MO, USA) in concentrations ranging from 0.05–5 μg/ml and 7-DHC (Santa Cruz Biotechnology, Inc., TX, USA) from 0.05–3.5 μg/ml for the standard curves [13]. The limit of detection was determined by measuring replicates of a blank sample, determining the mean and standard deviation (limit of detection = mean + 2 standard deviations). The limit of quantification was determined by measuring replicates of progressively more dilute concentrations of analyte, down to the lowest standard of 0.05 μg/ml, until the lowest concentration with a coefficient of variation of less than or equal to 10% was determined. The limit of detection for vitamin D_3 was determined to be 0.03 μg/ml, and limit of quantification 0.08 μg/ml. The intra-assay coefficient of variation was 6.5%. Results are reported as absolute values and as mean ± SD.

Results

Reverse phase HPLC revealed the presence of 7-DHC in skin samples of both the sheep and horses included in this study. The mean concentration of 7-DHC in ovine skin was 1.8 ± 0.3 μg/g (1.44 μg/cm^2, 3.75 nmol/cm^2), and in equine skin 5.0 ± 3.3 μg/g (3.3 μg/cm^2, 8.3 nmol/cm^2).

Ovine skin irradiated for 8 hr with 5 J/cm^2 of ultraviolet light contained 0.16 ± 0.07 μg/g vitamin D_3. The concentration of vitamin D in equine skin before and after irradiation with 5 J/cm^2 of ultraviolet light was below the detection limit of the assay (<0.03 μg/g) (Table 1). Figure 1 illustrates the presence of a peak consistent with vitamin D in sheep 1, but lack of a peak at the appropriate retention time for horse 1.

Table 1. Skin results

Species	Vitamin D_3 (μg/g) before ultraviolet light	Vitamin D_3 (μg/g) After 8 hr ultraviolet light
Sheep 1	<0.03	0.21
Sheep 2	<0.03	0.10
Horse 1	<0.03	<0.03
Horse 2	<0.03	<0.03
Horse 3	<0.03	<0.03
Horse 4	<0.03	<0.03
Horse 5	<0.03	<0.03

The concentration of vitamin D_3 (μg/g) in the ovine and equine skin samples before and after irradiation with 5 J/cm^2 (8 hr) ultraviolet light.

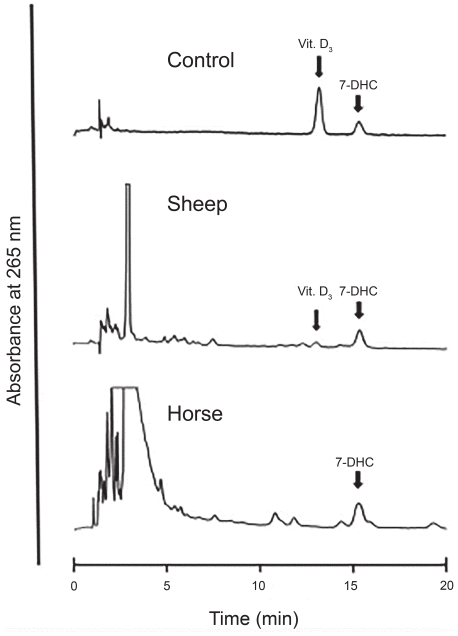

Fig. 1. Reversed-Phase chromatography HPLC analysis of the skin of sheep and horse (7-DHC: 7-dehydrocholesterol and Vit. D_3: vitamin D_3). A peak consistent with vitamin D is present in the sheep, but not in the horse.

Discussion

Ultraviolet radiation did not cause a detectable increase in the concentration of vitamin D_3 in skin samples from horses. This result, together with studies showing that 25-hydroxyvitamin D_2 is the predominant form of vitamin D in plasma in horses at pasture, and that 25-hydroxyvitamin D_3 is undetectable [1, 2], provides additional evidence to suggest horses produce negligible quantities of vitamin D_3 in their skin under the influence of ultraviolet radiation.

The results obtained from ovine skin samples in the present study showed that irradiation and exposure time to UVB light allowed conversion of 7-DHC to vitamin D_3, and that this conversion was measurable using the technique described. As no detectable increase in the amount of vitamin D_3 in the skin samples of the horses occurred, it is likely that horses do not make measurable amounts of vitamin D_3 in the skin. However we cannot rule out that a very small amount of vitamin D_3 was produced that was below the detection limit of the assay (<0.03 μg/g).

The mean concentration of 7-DHC in the equine skin samples (3.3 μg/cm^2 = 8.3 nmol/cm^2) was higher than that reported in dogs (1.86 ug/cm^2) and cats (1.96 ug/cm^2), while the mean 7-DHC concentration in ovine skin (1.44 ug/cm^2 = 3.75 nmol/cm^2) was lower than that reported in dogs and cats, but higher than polar bear skin (0.11 nmol/cm^2) [11, 16, 20]. The results of the present study, along with studies performed by others on cats and dogs, [11, 20], suggest that the presence of 7-DHC in skin does not guarantee it will be converted to vitamin D_3. In cats, lack of vitamin D_3 skin production was reversed by giving an inhibitor of the 7-dehydrocholesterol-D_7-reductase [20].

That horses appear to rely on dietary vitamin D_2 to fulfil their vitamin D requirements is thus far a unique finding. While there appear to be multiple evolutionary adaptations to relying on dietary vitamin D_3 [11, 16], to the authors' knowledge no animal species has been found to rely on dietary vitamin D_2. The evolution of the domesticated horse (*Equus ferus caballus*) saw a change in diet from jungle foliage in the early species to grasses as horses evolved in size and developed dentition that allowed a change from browsing to grazing [24]. *Equus* have other unique evolutionary features such as the greatest decrease in digit number, this allowed an increase in limb length, and a gait that allowed movement across the grasslands of the steppe regions of Europe and Asia [22]; similar to the evolution of the last surviving wild horse, the Przewalskii or Mongolian wild horse (*Equus ferus przewalskii*) [24]. Vitamin D

deficiency is common in humans in Central Asia due to inadequate solar radiation [7, 21]; as such perhaps this lack of solar radiation encouraged an adaptation for horses to obtain vitamin D from their diet that other herbivores elsewhere may not have required. In a similar manner, polar bears (*Ursus maritimus*), also from areas with long periods of inadequate solar radiation, are hypothesised to not make vitamin D_3 in their skin due to low dermal 7-DHC concentrations, and have evolved to rely on their diet for vitamin D requirements [16].

Further research into equine dermal vitamin D_3 production should examine different breeds of horse, and horses without skin pigmentation. The horses used in this study were all Thoroughbreds, two bay and three brown, all with significant skin pigmentation. Melanin acts as a natural photon absorber to decrease ultraviolet light penetration to the skin [4] and perhaps the lack of vitamin D production found in the skin of horses in this study is related to the melanin content of the skin. However, this does not completely explain the lack of vitamin D production as New Zealand sealions also have significant skin pigmentation and were found to produce large amounts of vitamin D_3 in their skin [15]. Future work will need to examine the mechanism behind the undetectable vitamin D production in horse skin.

In conclusion, the results of this study suggest that horses are unable to convert 7-DHC to vitamin D_3 in their skin after exposure to UVB light. This finding indicates that horses rely mainly on their diet as a primary source of vitamin D and the requirements of vitamin D in the equine diet should be reassessed.

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