Effects of dietary supplementation with synthetic vitamin D₃ and 25-hydroxycholecalciferol on blood calcium and phosphate levels and performance in laying hens

Einfluss von 25-Hydroxycholecalciferol vs. synthetischem Vitamin D₃ im Futter auf Calcium- und Phosphatwerte und Leistung bei Legehennen

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Introduction

Bone fractures are a widespread problem in commercial laying hens once hens enter the laying phase (Fleming et al., 2004; Sandländers et al., 2009). As the incidence of bone fractures closely correlates with egg laying, calcium metabolism could be a causal factor. Cholecalciferol (vitamin D₃) plays an important role in the calcium metabolism of the laying hen (Fleming et al., 2006; Rennie et al., 1997; Whitehead and Fleming, 2000). After oral uptake, dietary vitamin D₃ undergoes intestinal absorption and biochemical processing yields the active metabolite 1,25-dihydroxycholecalciferol. This conversion results from two consecutive steps. In the liver, cholecalciferol is first hydroxylated to 25-hydroxycholecalciferol (25(OH)D₃) by the enzyme 25-hydroxylase. Thereafter, an additional hydroxyl group is added by the renal 1-alpha-hydroxylase thus producing 1,25-dihydroxycholecalciferol, the biologically active form.

In poultry feeds, 25(OH)D₃ is commercially available as Hy•D⁺ (DSM Nutritional Products, Inc, Delft, The Netherlands). 25(OH)D₃ provides a means to bypass the first bio-transformation step in the liver and, consequently, the metabolic potency of 25(OH)D₃ was reported to be greater in broiler chicks than with vitamin D₃ uptake (Fritts and Waldroup, 2003). Several studies in broilers reported beneficial effects of dietary supplementation with 25(OH)D₃ on weight gain (Fritts and Waldroup, 2003), hatchability of eggs (Hart et al., 1986; Sund et al., 1978) and tibial dyschondroplasia (Zhang et al., 1997). In turkeys, however, studies on morphological characteristics of different bones as well as strength indicators of tibias did not clearly support the contention that the administration of 25(OH)D₃ improved the mineralization of leg bones (Faruga et al., 2009). With respect to chicks, 25(OH)D₃ is also recognized as the major vitamin D metabolite in blood (Haussler and Rasmussen, 1972). However, little is known about potential benefits of feeding 25(OH)D₃ to laying hens. One study reports that this vitamin D metabolite has a favorable effect on eggshell quality, bone metabolism and general performance (Soares et al., 1995). These authors also suggest 25(OH)D₃ to be an excellent marker for ascertaining the vitamin D status of poultry. By contrast, Keshavarz (2003) was unable to substantiate any positive effect of 25(OH)D₃ on shell quality or production performance in laying hens.

The present study in laying hens was conducted to investigate possible effects of a combined feeding of 25(OH)D₃ and synthetic Vitamin D₃ as compared with a control diet containing synthetic Vitamin D₃ only. Parameters included calcium, 25(OH)D₃ and phosphate levels in blood serum as well as performance data.

Materials and Methods

In total, 4000 LSL-chicks were reared from day one in eight pens of 500 animals each in a rearing aviary house with elevated perches, automatic chain-feeders, nipple drinkers and a litter area of about 32% of the total floor space. The pullets were fed a commercial starter diet (11.8 MJ ME, 20% CP) and pullet feed (11.6 MJ ME, 15.5% CP) which were supplemented in the four control pens with synthetic Vitamin D₃ only. In the four experimental pens, 25(OH)D₃ was added to the commercial feed (Table 1). All feeds were produced by the same feed mill. Raw nutrients (raw protein, lipids, ash, fat, fibre) and trace minerals (copper, iron, manganese, zinc) were checked at the laboratory of ALP, Pоесieu. Lighting and temperature regimes are given in Table 2.

At the age of five weeks, an opening between two neighboring pens resulted in a mixup of chicks. The total number of animals which switched pens remained unknown. Thereafter, all chicks were tagged with leg bands of specific colours and no further mixing occurred. Data of affected pens were not incorporated into the analysis of the rearing data except for the blood analyses.

At the age of 18 weeks, the pullets were transferred to the layer house. 2860 LSL- hens were kept in 8 pens with 355 to 360 animals per pen in two different aviary systems. The hens were fed a commercial layer feed phase 1 (11.6 MJ ME, 17.6% CP) and from 43 weeks of age layer feed phase 2 (11.4 MJ ME, 16.7% CP) which were again supplemented with vitamin D₃ and Hy•D⁺ (Table 1). At the age of eleven, eighteen, and thirty-four weeks, thirty to forty hens from each feed treatment were chosen randomly and...
2 ml of blood were collected from the jugular vein. The blood was withdrawn with a syringe and sterile needle and then transferred into a coagulation tube. Within three hours, the blood samples were transported to the laboratory in an insulated styropor box containing cooling elements. Samples were centrifuged at 2500 rpm for 15 minutes at room temperature prior to serum analysis. The total calcium and total inorganic phosphate serum levels were determined using commercial assay kits in a multi-titer plate format (Nr. 11730240 and Nr. 10759350, Roche Diagnostics AG Switzerland). The method was adapted to the multi-titer plate reader Spectra MAX 190 (Molecular Devices Inc. USA). As a control, the test sera ‘Precinorm’ and ‘Precipath’ were analyzed simultaneously (Roche Diagnostics AG Switzerland). Serum concentrations of 25-hydroxyvitamin D3 were determined with a commercial assay kit (CTL assay K2109, ImmunDagnostik AG, Bensheim). To assess the reliability of the measurements, total serum calcium levels were determined in blood samples from 18 LSL hens and two LSL males of different ages by two different laboratories. In the reference laboratory, plasma calcium was measured using an automated analyzer (Roche-Hitachi 912, Roche Diagnostics AG, Switzerland) according to the manufacturer’s instructions. In this laboratory, measurements were duplicated by splitting seven samples into two different coagulation tubes.

During rearing, feed intake and total mortality were assessed. Chicks were weighed daily by the automatic weighing system fancom 747. During the laying period, total mortality was also assessed. The number of eggs was recorded daily, every second week eggs were weighed. At the age of 49 and 66 weeks, egg shell breaking strength and shell thickness were measured with a breaking strength measuring instrument (BMG 1.2mc/D, Fabr.Nr. MC 601/047, Messgerätebau Gutsch, Nauendorf) and egg shell thickness with an egg piece of the egg equator with an “IBA” dial gauge. Ten hens per pen were weighed at the age of 20, 45 and 66 weeks individually with a Mettler Toledo Viper SW balance.

Keel bone status of ten hens per pen was assessed at the age of 65 weeks. The same investigator (S.K) performed all examinations by catching a hen and palpating the keel bone by running two fingers down the edge of the keel bone to feel for alterations like s-derivations, bumps or depressions.

### Statistical Analysis

Data and residuals were checked for normality, data were checked for homogeneity of variances. Calcium values were transformed by taking logarithms. 25(OH)D3 values of ages 11 and 18 weeks were transformed by taking the reciprocal value. Blood data were analyzed by generalized linear mixed models separately for different ages because animal...
mals were housed in different pens after week 18. Pen (nested in feed) was used as a random effect. The ratio of calcium/phosphate was averaged within pens and a median test was performed. Analyses of blood data were done using SAS 9.1. Performance data were analyzed using the statistic program NCSS (Number Cruncher Statistical Systems of Dr. J.L. Hintze 2004) by a one-factorial analysis of variance (nested in feed) was used as a random effect. The ratio of calcium/phosphate was significantly higher in the Hy•D® group than in the control group (Table 6). Hens of the group Hy•D® consumed more feed by trend and their feed conversion ratio tended to be higher as compared with the consumption of control hens. Measuring egg shell breaking strength at trial’s end showed a higher breaking strength by trend and a thicker egg shell for eggs of the control group as compared with the Hy•D® group. The prevalence of keel bone fractures did not differ significantly between feeding regimens (Table 6).

Results

Blood parameters

Feeding Hy•D® led to a significantly higher concentration of serum 25(OH)D$_3$ at weeks 18 and 34 (Table 3). At the age of 11 weeks, chicks fed with Hy•D® tended to have a higher concentration of serum 25(OH)D$_3$ (P = 0.0545) (Table 3). No difference in the serum concentration of total calcium was observed between the treatment groups in any of the measurements. Serum calcium levels increased significantly from week 18 to week 34, rising from 3 mmol/l in both treatment groups to 8.32 mmol/l for the control group and to 8.66 mmol/l for the treatment group, respectively (Table 3). At the ages of 11 and 34 weeks, phosphate concentration was significantly higher in the Hy•D® group. The prevalence of keel bone fractures did not differ significantly between feeding regimens (Table 6).

Reliability

The comparison of the two laboratories which analyzed the same twenty samples showed a systematic difference of about 1 mmol/L (F$_{1,34}$ = 10.2, P = 0.003, N = 18) (Table 4). The correlations between the two laboratories ($r_p$ = 0.98, $N$ = 20, $P < 0.0001$) and also between the two tubes in one laboratory were very high ($r_p$ = 0.99, $N$ = 8, $P < 0.0001$).

Table 3. Serum levels of total calcium, phosphate, and 25(OH)D$_3$. Means are shown with standard deviations. The sample sizes are given in parentheses for calcium values and apply to all measurements. Different subscripts indicate $p < 0.05$.

Gleichzeitige Analysen des Calciums im Serum von Legehennen von zwei verschiedenen Laboratorien.

<table>
<thead>
<tr>
<th>Age [weeks]</th>
<th>Calcium [mmol/l]</th>
<th>Phosphate [mmol/l]</th>
<th>25(OH)D$_3$ [mmol/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hy•D</td>
<td>Control</td>
</tr>
<tr>
<td>11</td>
<td>2.67 ± 0.34 (30)</td>
<td>2.51 ± 0.12 (30)</td>
<td>1.98 ± 0.16</td>
</tr>
<tr>
<td>18</td>
<td>3.01 ± 0.13 (40)</td>
<td>3.01 ± 0.10 (35)</td>
<td>1.70 ± 0.21</td>
</tr>
<tr>
<td>34</td>
<td>8.32 ± 1.20 (40)</td>
<td>8.66 ± 1.13 (40)</td>
<td>1.80 ± 0.25</td>
</tr>
</tbody>
</table>

Table 4. Analyses of serum calcium levels [mmol/l] of laying hens of the same blood samples by two different laboratories at the same time.

Table 5. Body weight, feed consumption and mortality of LSL pullets at the age of 16 weeks. Means are shown with standard deviations.


<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight kg</th>
<th>Feed consumption kg</th>
<th>Mortality %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.21 ± 0.01</td>
<td>5.08 ± 0.14</td>
<td>2.57 ± 0.01</td>
</tr>
<tr>
<td>Hy•D</td>
<td>1.17 ± 0.06</td>
<td>5.03 ± 0.01</td>
<td>2.47 ± 0.01</td>
</tr>
<tr>
<td>Significances</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Performance

The pullets reached a body weight of 1.19 kg on average (Table 5). No significant differences were noted between treatments. Laying rate over the whole laying period resulted in over 90% and was slightly though not significantly higher for hens fed Hy•D® as compared with hens of the control group (Table 6). Hens of the group Hy•D® consumed more feed by trend and their feed conversion ratio tended to be higher as compared with the consumption of control hens. Measuring egg shell breaking strength at trial’s end showed a higher breaking strength by trend and a thicker egg shell for eggs of the control group as compared with the Hy•D® group. The prevalence of keel bone fractures did not differ significantly between feeding regimens (Table 6).

Discussion and conclusion

The main results of the present study comprise increased serum 25(OH)D$_3$ concentrations after Hy•D® supplementation, a substantial rise in serum calcium levels with the onset of laying irrespective of vitamin D supply and a possible dietary effect of Hy•D® on the serum phosphate level. A clear difference between Hy•D® and control groups was found with respect to serum 25(OH)D$_3$ levels. Replacing half the dose of the vitamin D$_3$ in the diet with 25(OH)D$_3$ resulted in a significantly higher serum level of the hydroxylated form. The falling short of significance at age 11 might be due to the incident at the age of five weeks when mixing up of chicks occurred. Since hens were still growing, this might have influenced the serum levels. Despite the significant effects of Hy•D® on 25(OH)D$_3$ levels, no effect on
Table 6. Production parameters of laying hens with the control and the Hy•D enriched feeds. Number of eggs per hen housed, average laying rate, feed per hen, feed per egg, feed conversion ratio (FCR, kg feed/kg egg mass), egg weight were taken when hens were between 21 and 68 weeks of age. Means are shown with standard deviations. Body weight was taken at week 68, the mortality is cumulative between weeks 21–68. The percent of hens with broken keel bones was investigated at the age of 65 weeks.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Hy•D</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight [g]</td>
<td>1835 ± 180</td>
<td>1767 ± 229</td>
<td>N.S.</td>
</tr>
<tr>
<td>Mortality [%]</td>
<td>12.29 ± 0.09</td>
<td>13.83 ± 0.09</td>
<td>N.S.</td>
</tr>
<tr>
<td>Eggs per hen</td>
<td>286 ± 17.7</td>
<td>285 ± 10.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>Average laying rate</td>
<td>90.4 ± 0.01</td>
<td>91.1 ± 0.01</td>
<td>N.S.</td>
</tr>
<tr>
<td>Feed per hen per day [g]</td>
<td>114 ± 2.06</td>
<td>115 ± 3.14</td>
<td>N.S.</td>
</tr>
<tr>
<td>Feed per egg [g]</td>
<td>126 ± 1.87</td>
<td>127 ± 2.37</td>
<td>N.S.</td>
</tr>
<tr>
<td>FCR [kg/kg]</td>
<td>1.98 ± 0.03</td>
<td>1.98 ± 0.03</td>
<td>N.S.</td>
</tr>
<tr>
<td>Egg weight [g]</td>
<td>63.8 ± 0.36</td>
<td>63.8 ± 0.39</td>
<td>N.S.</td>
</tr>
<tr>
<td>Egg shell thickness [1/100 mm]</td>
<td>38.3 ± 2.08</td>
<td>37.8 ± 2.66</td>
<td>N.S.</td>
</tr>
<tr>
<td>Egg shell breaking strength [N]</td>
<td>39.3 ± 8.29</td>
<td>37.2 ± 9.49</td>
<td>N.S.</td>
</tr>
<tr>
<td>Broken keel bones [%]</td>
<td>30.0 ± 13.9</td>
<td>40.0 ± 15.4</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Production parameters was detected. Reports on the effects of dietary 25(OH)D₃ supplementation are contradictory. In an experiment with broiler breeders, ATENCIO et al. (2005) observed that a complement of 25(OH)D₃ improved the hen-day egg production but only at very low levels of overall vitamin D supplementation. In quails, the addition of dietary 25(OH)D₃ increased final body weight, serum vitamin D levels, and it also improved feed conversion. Together with soy isoflavones, the addition of dietary 25(OH)D₃ increased bone mineral density, bone ash, Ca, P, and serum vitamin D levels (SAHIN et al., 2009). FRITTS and WALDROUP (2003) reported that 25(OH)D₃ supplementation significantly reduced the incidence and severity of tibial dyschondroplasia. This outcome, however, was observed whenever vitamin D uptake was increased, i.e. regardless of the source of vitamin D. Moreover, beneficial effects were observed primarily in ranges of low vitamin D supply. Differences disappeared when control groups were fed according to industrial raising standards. As in NARBATZ and TOLNAI (1978, cited in SOARES et al., 1995) and KESHA-VAZ (2003) we did not observe improved shell quality or production performance in laying hens. Neither did the increase in 25(OH)D₃ serum levels have beneficial effects on fructures of the keel bones. In order to determine whether keel bone and bone quality in general can be improved by 25(OH)D₃ supplementation, further studies are needed.

As seen in Table 1, the concentration of calcium in the starter control feed was lower than in the Hy•D diet. Since the reference value was correctly added in the feed, there may have been a problem in the analyses due to imperfect mixing.

A significantly higher serum calcium concentration was observed in week 34 during the laying period. Required adjustments of calcium metabolism to egg shell production provide a straightforward rationale for this change. In white Leghorn hens, CHARLES and HOGBEN (1933) observed that a functionally active ovary maintains the blood calcium at a higher level than in sexually immature birds. Blood calcium levels were 2.85 mmol/l in sexually immature birds and 4.25 mmol/l in laying hens during the interval between oviposition and subsequent ovulation. These data are fully compatible with the measurements presented here. However, this does not take into account the considerable fluctuations in serum calcium levels in the course of a day. Thus, the presence of an egg in the oviduct with accompanying shell secretion goes along with calcium mobilization from the skeleton, thereby producing a transient short-term rise in blood calcium. Calcium and phosphate serum levels thus depend on the current status of egg formation and are not constant throughout the day (CHOI et al., 1979). Circadian variation was not taken into account in the present study. However, blood samples were collected at the same time of day for all animals when all eggs had been laid, thus eliminating a possible sampling bias.

Our results demonstrate that dietary 25(OH)D₃ supplementation does not affect serum calcium levels. This is likely to be due to the strict homeostatic control of the conversion of 25(OH)D₃ to 1,25-dihydroxycholecalciferol in order to maintain adequate serum calcium levels (CHOI et al., 1979). Whether dietary 25(OH)D₃ supplementation truly lowers blood phosphate concentrations as observed at weeks 11 and 34 remains uncertain as the pattern of phosphate metabolism is closely related to egg production. CHOI et al. (1979) reported that both total serum calcium and inorganic phosphate levels rise during shell formation. Whereas the serum calcium level quickly returns to normal due to calcium removal from the circulation by the shell gland, the gradual adjustment of serum phosphate concentrations by renal excretion lags behind. Therefore, serum phosphate concentrations drop to the lowest level of the daily cycle several hours after egg shell calcification is completed at a time when bone mineralization prevails again. Thus, variations in serum phosphate concentrations are not synchronous to the serum calcium pattern. As the time of blood sample collection relative to egg formation was not taken into account in the different age groups, the divergence in phosphate levels observed in the present study may simply reflect a sampling bias due to differences in the schedule of shell formation. To unequivocally assess
a possible effect of dietary 25(OH)D$_3$ supplementation on blood phosphate concentrations, repeated daily blood sampling would be needed.

In conclusion, the present study demonstrates that 25(OH) serum levels can be elevated by partially substituting regular vitamin D$_3$ supply with 25(OH)D$_3$ in the diet. As few data are available on typical serum calcium levels and 25(OH) D$_3$ levels in laying hens, our results also provide a basis for further studies on blood characteristics regarding the calcium metabolism in laying hens.

Acknowledgements

We thank the staff of the Aviforum for taking care of the hens. Hans Oester’s suggestions and the comments of two anonymous reviewers substantially improved the manuscript. We acknowledge the financial support of the Federal Veterinary Office of Switzerland and DSM.

Summary

We investigated the effects of different dietary vitamin D regimen on selected blood parameters in laying hens. Supplementation with vitamin D$_3$ only was compared with a combination of vitamin D$_3$ and its metabolite 25-hydroxycholecalciferol (25(OH)D$_3$). Blood concentrations of total calcium, phosphate and 25(OH)D$_3$ were determined. Four thousand one-day-old LSL chicks were split in two treatment groups and distributed to eight pens. The control group was given a commercial animal diet containing 2800 IU synthetic vitamin D$_3$ in the starter feed and 2000 IU synthetic vitamin D$_3$ in the pullet feed. The experimental group was fed the same commercial diet in which half the synthetic vitamin D$_3$ content had been substituted with 25(OH)D$_3$ (Hy•D®). At 18 weeks of age, pullets were transferred to the layer house. At the ages of 11, 18 and 34 weeks, between 120 and 160 blood samples were collected from both the control and the experimental groups, respectively. The experimental group had higher levels of 25(OH)D$_3$ than the control group at all three ages. Serum calcium levels did not differ between the treatment groups at any age. With the onset of laying, calcium levels rose significantly. Whereas blood serum concentration at 18 weeks was 3 mmol/L in both treatment groups, it increased to 8.32 mmol/L in the control group and to 8.66 mmol/L in the Hy•D® Futtergruppen and 8.66 mmol/L in the Hy•D® Futtergruppen in Woche 34 an. In the Wochen 11 and 34 were the Phosphatwerte in den Kontrollfuttergruppen signifikant höher als in den Hy•D® Gruppen. Die Hy•D® Gruppen hatten signifikant höhere 25(OH)D$_3$ Werte in allen drei Untersuchungen. Zusammenfassend kann man sagen, dass die Fütterung von Hy•D$^*$ das Serum 25(OH)D$_3$ und das Serum Phosphat signifikant beeinflusste. Die Legeleistung, Schalenqualität und Brustbeinfrakturen wurden durch die Zugabe von Hy•D$^*$ nicht beeinflusst.

Stichworte

Vitamin D$_3$, Calcium, 25-Hydroxycholecalciferol, Phosphat, Legehennen

References


CHARLES, E., L. HOGген, 1933: The serum calcium and magnesium level in the ovarian cycle of the laying hen. Exp. Physiol. 23, 343-349.


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