The effect of dietary 1,25-dihydroxycholecalciferol in preventing tibial dyschondroplasia in broilers fed on diets imbalanced in calcium and phosphorus

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Three experiments were carried out to investigate the effects of supplemental dietary 1,25-dihydroxycholecalciferol (1,25(OH)$_2$cholecalciferol) and a low dietary Ca:P ratio on the occurrence of tibial dyschondroplasia (TD) in 3-week-old broilers. Histopathology was used to diagnose TD. In the first experiment, feeding a diet containing 7·5 g Ca and 7·6 g P/kg gave a higher incidence of TD than a control diet containing normal amounts of Ca and P (12 and 6 g/kg respectively). Increasing the dietary supplement of cholecalciferol in the imbalanced diet prevented rickets but did not decrease the incidence of TD. In the second experiment, supplementing the imbalanced diet with 10 μg 1,25(OH)$_2$cholecalciferol/kg prevented TD completely but also gave a slight growth depression. In the third experiment the imbalanced diet was supplemented with 0, 2·5, 5 or 10 μg 1,25(OH)$_2$cholecalciferol/kg. The supplement of 2·5 μg/kg depressed and the higher supplements prevented the occurrence of TD, this time without a growth depression. Feeding the 10 μg/kg supplement for the first week only did not prevent TD. Plasma total Ca, inorganic P and alkaline phosphatase (*EC* 3.1.3.1) were unaffected by diet but 1,25(OH)$_2$cholecalciferol was higher on the imbalanced than on the control diet. Supplementation of the imbalanced diet with 1,25(OH)$_2$cholecalciferol did not increase plasma levels. It is concluded that 1,25(OH)$_2$cholecalciferol is exerting a powerful biological effect in this model of TD, but the mechanism is unclear.

Tibial dyschondroplasia: Cholecalciferol: 1,25(OH)$_2$cholecalciferol: Calcium–phosphorus balance: Broilers

Tibial dyschondroplasia (TD) is a well-recognized disorder of rapidly growing broilers, first described by Leach & Nesheim (1965). TD affects the long bones, particularly the proximal tibiotarsus (PTT), and is characterized by the presence there of an avascular plug of cartilage. If the lesion is large, affected birds may become lame due to weakening, bowing or fracture of the PTT (Lynch *et al.* 1991). Less severely affected birds show thickening of the growth plate. Estimates of clinically affected birds are as high as 10 % and many more birds may be sub-clinically affected.

Histologically the lesion consists of a mass of pre-hypertrophied chondrocytes, due to a failure, for as yet unknown reasons, of the normal sequence of chondrocyte differentiation through proliferating, hypertrophying and calcifying stages (Farquarson *et al.* 1991).

A high incidence of TD can be induced experimentally in a number of ways: for example, feeding diets with an altered cation:anion ratio or acid–base balance (Leach & Nesheim, 1972; Halley *et al.* 1987), fusarochromanone administration, or by feeding certain organic sulphur compounds (Vargas *et al.* 1983; Veltmann *et al.* 1985; Edwards, 1987). Edwards & Veltmann (1983) reported that feeding diets imbalanced in Ca–P could induce a high

incidence of TD as assessed by sectioning of bone extremities and gross examination of the growth plate. However, Riddell & Pass (1987), using histological techniques, found that chickens fed on diets containing high concentrations of P developed thickened growth plates identical to those seen in Ca-deficiency rickets before the development of typical TD.

An involvement of cholecalciferol metabolites in TD has been reported (Edwards, 1989, 1990). Using basal diets low in Ca (7·5 g/kg) and high in P (7·6 g/kg), the addition of 1,25-dihydroxycholecalciferol (1,25(OH)$_2$cholecalciferol) to a diet containing supplemental cholecalciferol in the presence and absence of disulfiram was found to result in a considerable increase in bone ash and a decreased incidence and severity of TD. However, in none of these experiments was this or any other metabolite of cholecalciferol found to prevent TD.

In these studies (Edwards, 1989, 1990) the occurrence of TD was assessed by visual examination of slices of PTT and identification of thickening of the growth plate. However, naked eye identification of TD is not so reliable as histopathological methods. This is because thickening of the growth plate can occur in the absence of cellular changes characteristic of TD (Thorp *et al.* 1991). Visual identification of TD lesions would be complicated further if rickets were also induced by the feeding of Ca–P imbalanced diets, as rickets will also cause thickening of the growth plate.

The aim of the present study was to carry out a detailed histological investigation of the effects on feeding 1,25(OH)$_2$cholecalciferol on the occurrence of TD and rickets in young broilers fed on diets containing an imbalance of Ca and P. Diagnosis of growth plate lesions was based on histopathology and biochemical characteristics of birds were also assessed.

MATERIALS AND METHODS
Birds and husbandry

Three experiments were conducted using groups of 1-d-old broiler chicks (Cobb strain; H. Morrison, Duns, Berwickshire) reared to 3 weeks of age in wire-floored electrically-heated brooder units. Food and water were available *ad lib.* and diets, based on wheat and soya-bean meal, were either a control diet calculated to contain (g/kg) Ca 12, P 6, or a TD-inducing diet calculated to contain (g/kg) Ca 7·5 and P 7·6 (Table 1). The TD-inducing diet was supplemented with various amounts of 1,25(OH)$_2$cholecalciferol, 0·2 g/kg in a stable matrix, kindly donated by Dr H. Weiser, Hoffmann–La Roche, Basle.

The aim of Expt 1 was to confirm, using histology, that diets imbalanced in Ca–P could induce a high incidence of TD, and to test the effects of giving birds extra cholecalciferol. Three groups of birds were used, the first receiving the control diet and the second and third groups receiving the TD-inducing diet containing either 25 or 75 μg cholecalciferol/kg.

Expt 2 assessed the effects of dietary supplementation of the TD-inducing diet with 1,25(OH)$_2$cholecalciferol. Three groups were again used, the first receiving the control diet, the second the TD-inducing diet, and the third the TD-inducing diet containing 10 μg added 1,25(OH)$_2$cholecalciferol/kg.

Expt 3 assessed the effects of supplementing the TD-inducing diet with various amounts of 1,25(OH)$_2$cholecalciferol, and consisted of a control group and four experimental groups receiving the TD-inducing diet supplemented with either 0, 2·5, 5, or 10 μg 1,25(OH)$_2$ cholecalciferol/kg. A further group was fed the last diet up to 7 d of age before being transferred to the unsupplemented TD-inducing diet.

At 3 weeks of age birds were weighed and blood samples were taken from the wing vein of ten to fifteen birds per treatment, using heparin as anticoagulant. Plasma was separated and stored at $-20°$ until it could be analysed. After blood sampling, all birds were killed by cervical dislocation and the PTT was examined and scored visually for growth plate

Table 1. *Composition of experimental diets (g/kg)*

Ingredient	Control diet	Imbalanced diet
Wheat	559	563
Soya-bean meal	350	350
Vegetable oil	45	50
Limestone	13	—
Dicalcium phosphate	21	25
Salt	4	4
Methionine	2	2
Lysine	2	2
Vitamin and mineral supplement*	4	4
Calculated analyses (g/kg)		
Ca	12	7·5
P	6	7·6

* Provided (mg/kg diet): retinol 1376 μg, cholecalciferol 25 μg, α-tocopherol 10, menadione 1·3, thiamine 1·3, riboflavin 3·3, pyridoxine 1·7, cyanocobalamin 0·6 μg, folic acid 0·7, biotin 267 μg, pantothenic acid 5·3, nicotinic acid 23·3, zinc 40, copper 2·9, iodine 320 μg, iron 64, manganese 80, selenium 120 μg.

thickening. Sections of PTT were taken for histological examination to confirm TD and to characterize growth plate pathology.

Laboratory procedures

Total Ca, inorganic phosphate and alkaline phosphatase (*EC* 3.1.3.1) were measured using Wako kits (Alpha Laboratories, Eastleigh, Hants.) adapted for use with a plate-reader (Titertek Twin-Reader Plus; ICN, High Wycombe, Bucks.). Total Ca was measured in undiluted plasma, using 4 μl plasma, 200 μl buffer and 20 μl colour reagent. A standard curve was prepared over the range 0–5 mM by dilution with extra-pure water (Millipore, Watford, Herts.) of standards provided with the kit. Samples were read after 20 min at 560 nm. Inorganic phosphate was also measured in undiluted plasma, using 5 μl plasma and 125 μl mixed colour reagent. A standard curve was again constructed by dilution of standards provided with the kit over the range 0–100 mg/l, and samples read after 20 min at 690 nm. Alkaline phosphatase was measured in plasma diluted eighty times with extra-pure water. Samples were kept on ice until they could be assayed. A standard curve was prepared by diluting 5 mM-*p*-nitrophenol (Sigma, Poole, Dorset) in assay buffer to give a range of standards from 0 to 62·5 μM. Enzyme activity was measured by adding 10 μl diluted plasma to 250 μl substrate, and measuring absorbance at 405 nm for 10 min. The mean change in absorbance per min was calculated and related to *p*-nitrophenol concentration. Alkaline phosphatase activity was calculated after correction for dilution, and expressed as units (U). 1,25(OH)$_2$cholecalciferol was measured in plasma using an Incstar kit (Incstar Ltd, Wokingham, Berks.).

Samples of PTT were fixed in buffered neutral formalin before decalcification in formic acid–formalin. Paraffin embedded sections were then prepared and stained with haematoxylin and eosin and examined. TD was diagnosed when characteristic changes to the chondrocytes of the proliferating, pre-hypertrophied and hypertrophied zones were seen, including accumulation of avascular matrix and degenerative changes in cytoplasm and nuclei. Rickets was diagnosed where changes in the growth plate included a widening of the proliferating zone and disturbances in vascularity within the growth plate (Lacey & Huffer, 1982).

Statistical analyses

Data were analysed by analysis of variance using Genstat statistical program.

RESULTS

The results from the first experiment confirm that feeding diets imbalanced in Ca and P to birds can induce a high incidence of TD (Table 2). The control group had a 14% incidence of TD, as assessed histologically, compared with a 21% incidence of TD and a 14% incidence of rickets in the group fed on the imbalanced diet containing 25 μg cholecalciferol/kg, and a 46% incidence of TD and 0% incidence of rickets in the group fed on the imbalanced diet containing 75 μg cholecalciferol/kg. There were no significant differences between treatments in bird weights, or between plasma Ca, P or 1,25(OH)$_2$ cholecalciferol concentrations. Alkaline phosphatase measurements were unsuccessful in the first experiment as the samples were not sufficiently diluted.

The second experiment showed that addition of 1,25(OH)$_2$cholecalciferol to a diet imbalanced in Ca and P can reduce the incidence of TD. The control group had a 29% incidence of TD compared with 19% in the group fed on the imbalanced diet. The incidence dropped to zero in the group fed on the imbalanced diet supplemented with 10 μg 1,25(OH)$_2$cholecalciferol/kg. There was a significant difference in body weights between treatments, with the group receiving 1,25(OH)$_2$cholecalciferol having a significantly lower mean body weight than birds on the other two treatments. Again, there were no significant differences in plasma Ca, P or alkaline phosphatase between treatments.

The third experiment showed that the response to dietary 1,25(OH)$_2$cholecalciferol is influenced by dose, in that the group receiving no 1,25(OH)$_2$cholecalciferol had a 36% incidence of TD, and the groups receiving 2·5, 5, or 10 μg/kg had 19, 0, and 0% incidences respectively. In the present experiment there were no significant differences in body weights between treatments, although there was a trend towards lower body weight at the higher levels of supplementation of 5 and 10 μg 1,25(OH)$_2$cholecalciferol/kg. There were no significant differences in plasma Ca, P or alkaline phosphatase between treatments, although there was a trend towards hypercalcaemia with increasing dietary 1,25(OH)$_2$cholecalciferol. Feeding a diet imbalanced in Ca and P significantly increased mean circulating 1,25(OH)$_2$cholecalciferol levels in Expt 3 (although not in Expt 1) from 40 to 65 pg/ml. Concentrations increased from a mean of 49 pg/ml in birds receiving 2·5 μg 1,25(OH)$_2$ cholecalciferol/kg, to a mean of 67 pg/ml in birds receiving 10 μg 1,25(OH)$_2$chole-calciferol/kg.

DISCUSSION

These experimental results demonstrate that dietary supplementation with 1,25(OH)$_2$ cholecalciferol can prevent the occurrence of TD in broilers fed on a diet imbalanced in Ca and P. The present study extends the observations of Edwards (1989, 1990) who demonstrated in several experiments substantial decreases in the incidence of TD after dietary supplementation with this metabolite. The observations of complete prevention of TD at the higher supplementation rates in the present study are perhaps the result of the use of histology to make a specific diagnosis of TD in the presence of other growth plate abnormalities, including rickets, which were also found to occur with the experimental diets used.

The first experiment confirmed that the use of a diet imbalanced in Ca and P and containing 25 μg cholecalciferol/kg could give a higher incidence of TD than a control diet containing normal amounts of Ca, although this was not always the case in later experiments. That TD is not caused by a dietary deficiency of cholecalciferol *per se* was

Table 2. *Effects of different dietary calcium, phosphorus, cholecalciferol (D_3) and 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) concentrations on the growth, incidences of tibial dyschondroplasia and rickets, and plasma Ca and P in broilers*

(Mean values with their standard errors)

Expt no.	Ca (g/kg)	P (g/kg)	D_3 (μg/kg)	$1,25(OH)_2D_3$ (μg/kg)	Wt (g)			Percentage TD	Percentage rickets	Plasma concentrations											
										Ca (mM)			P (mg/l)			AP (U)			$1,25(OH)_2D_3$ (pg/ml)		
					Mean	SE	n			Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
1	12	6	75	0	476[a]	9.8	30	14	0	2.59[a]	0.19	15	72.2[a]	4.6	15	—	—		37[a]	3.4	8
	7.5	7.6	25	0	535[b]	13.2	30	21	14	2.99[a]	0.13	15	94.9[a]	6.0	15	—	—		42[a]	5.4	6
	7.5	7.6	75	0	533[b]	11.8	29	46	0	2.94[a]	0.16	15	83.7[a]	5.6	15	—	—		42[a]	4.9	10
2	12	6	25	0	647[a]	13.9	28	29	0	1.55[a]	0.06	25	57.6[a]	2.0	25	17329[a]	3093	25			
	7.5	7.6	25	0	650[a]	14.7	29	29	0	1.58[a]	0.06	17	53.6[a]	2.4	16	15080[a]	2274	17			
	7.5	7.6	25	10	552[b]	22.6	29	0	0	1.62[a]	0.06	26	60.6[a]	2.2	25	13136[a]	1877	26			
3	12	6	25	0	546[a]	15.5	17	0	11	2.18[a]	0.11	10	57.1[a]	2.1	10	17111[a]	1760	10	40[a]	4.0	10
	7.5	7.6	25	0	532[a]	19.1	17	36	0	1.90[a]	0.16	9	66.5[a]	2.9	9	17736[a]	2174	9	65[b]	7.8	9
	7.5	7.6	25	2.5	549[a]	9.7	16	19	0	2.07[a]	0.13	10	60.1[a]	2.5	10	18578[a]	2613	10	49[a]	5.6	9
	7.5	7.6	25	5	516[a]	17.9	19	0	0	2.19[a]	0.11	9	66.0[a]	3.0	10	17687[a]	3038	10	53[a]	5.6	9
	7.5	7.6	25	10	522[a]	23.8	16	0	0	2.37[a]	0.17	9	67.3[a]	2.9	9	13047[a]	2837	9	67[b]	7.4	9

AP, alkaline phosphatase (*EC* 3.1.3.1).

[a,b] Within a column, values having different superscript letters were significantly different ($P < 0.05$).

demonstrated by the fact that supplementation of the imbalanced diet to give a total cholecalciferol content of 75 μg/kg did not reduce the incidence of TD, although it did prevent rickets.

The second experiment showed that a supplement of 10 μg 1,25(OH)$_2$cholecalciferol/kg could prevent the TD induced in this model. However, there was a significant depression of body weight with this diet, and a lower rate of growth is known to be associated with a decreased incidence of TD (Huff, 1980). In broiler chicks fed *ad lib.*, growth accelerates rapidly from 2 weeks of age, with maximum allometric growth in the PTT occurring between 2 and 3 weeks of age (Thorp, 1988). Therefore, during this crucial period, any factor reducing growth rate will also reduce chondrocyte differentiation and, therefore, the likelihood of TD. The toxic effects of cholecalciferol metabolites were considered as a cause of the lowered body weight in this group, but as this result was not duplicated in the third experiment it was felt to be due instead to a factor outwith the experiment. The standard error of the mean for body weight was much higher in this group compared with other treatments.

The third experiment showed that a lower dose was also effective in reducing the incidence and severity of TD, and that dietary contents of 5 and 10 μg/kg were effective in preventing TD. In this experiment there was no significant growth depression at the higher supplemental levels. Plasma P and alkaline phosphatase were unaffected by supplementation whereas plasma Ca tended to be elevated, although the changes were not statistically significant. Circulating concentrations of 1,25(OH)$_2$cholecalciferol were elevated among birds given the TD-inducing diet compared with the control diet, but did not show any obvious response to dietary supplementation of the TD-inducing diet with 1,25(OH)$_2$ cholecalciferol. It is well known that Ca deficiency increases the renal synthesis of 1,25(OH)$_2$cholecalciferol (Cancela *et al.* 1988) and in a recent study (Goff *et al.* 1990) circulating concentrations achieved after dietary restriction were comparable with those seen after injection of 1,25(OH)$_2$cholecalciferol, both being approximately eight times normal. In the present study 1,25(OH)$_2$cholecalciferol was administered orally. Its efficiency of absorption is not known, but absorption must have occurred in view of the profound effect on the incidence of TD. However, plasma concentrations of 1,25(OH)$_2$ cholecalciferol are apparently not related to the development of TD.

It was also of interest in the third experiment that supplementation with 10 μg 1,25(OH)$_2$cholecalciferol/kg for the first week after hatching only did not prevent TD. This implies that supplementation is needed over the whole period when rapid bone growth is likely to make the growth plate more susceptible to TD. It also suggests that the problem does not originate at the breeder level, with inadequate carryover of cholecalciferol and its metabolites to the hatching chick.

It is clear from the experimental results that 1,25(OH)$_2$cholecalciferol is exerting a powerful biological effect in this TD model. *In vitro* studies have demonstrated receptors for 1,25(OH)$_2$cholecalciferol on chondrocytes (Suda *et al.* 1985) and the metabolite has been shown to have a powerful inhibitory effect on cell proliferation and a stimulatory effect on cell differentiation in chondrocytes derived from embryonic chicken caudal sterna (Gerstenfeld *et al.* 1990). A basis for the involvement of 1,25(OH)$_2$cholecalciferol, therefore, exists in this model of TD, either related to the supply of the metabolite to the chondrocyte and/or its effects on cellular activity. Soares *et al.* (1990) found a trend towards reduced numbers of 1,25(OH)$_2$cholecalciferol receptors in growth plate and intestine of TD-affected birds, but normal affinity for the hormone, as measured by Scatchard analysis. However, it is known from human studies (Marx, 1989) that defects related to cholecalciferol metabolism are complex, and may occur at every stage of the hormone acting on its target cell, from locating its receptor to binding to the nuclear DNA.

It is also possible that the observed response to 1,25(OH)$_2$cholecalciferol is mediated through a further metabolite of the hormone.

The role of 1,25(OH)$_2$cholecalciferol in preventing TD in this model is as yet uncertain, and its significance in field cases can only be the subject of speculation. Further *in vitro* and *in vivo* studies are necessary to elucidate the mechanism by which broilers develop TD, and to clarify the role of cholecalciferol metabolism in the occurrence of this important condition.

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