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Effect of Dexamethasone and Cyclophosphamide on Urinary Hydroxyproline to Creatinine Ratios in Sheep

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Abstract

The possibility of altering bone collagen turnover rates by injecting large doses of dexamethasone or by administering large doses of cyclophosphamide orally was investigated in sheep on low and high dietary intakes by measuring changes in the ratios of free and total hydroxyproline to creatinine and of calcium to creatinine in the urine. Dexamethasone at the rate of 1·15 mg/kg live weight caused an increase in free hydroxyproline excretion of 111 and 26% and in calcium excretion of 402 and 243% in the low and high intake sheep respectively. Cyclophosphamide (10 mg/kg) had little effect except for causing a slight decrease in total hydroxyproline excretion in the sheep on the high dietary intake. Heavier sheep on high dietary intakes excreted more free hydroxyproline than lighter sheep on low intakes.

It was concluded that dosing sheep with corticosteroids for the purpose of defleecing could cause abnormal collagen metabolism. Skeletal disorders are a likely result but further long-term field work would be necessary to determine the practical significance of these findings.

Introduction

Collagen contains nearly all the body's hydroxyproline and alterations in urinary hydroxyproline excretion reflect changes in the body's turnover of collagen (for reviews see Kivirikko 1970; Robins 1977). This should be viewed with some concern if it occurs as the result of corticosteroid doses at levels similar to those used for chemical defleecing purposes (Panaretto 1979) because (1) one of the undesirable side-effects of corticosteroid therapy in humans is the likelihood of inducing osteoporosis or other skeletal disorders (Janoski *et al.* 1968); (2) there is a relationship between bone turnover and hydroxyproline excretion. For example, Klein *et al.* (1961) found a correlation of 0·69 between calcium resorption rate and total hydroxyproline excretion in 33 humans. Black and Capen (1971) used hydroxyproline as an index of bone turnover rate in hypocalcaemic cows.

Although corticosteroids have shown promise as defleecing agents for sheep (Panaretto 1979) concomitant changes in collagen turnover leading, for example, to osteoporosis or other skeletal abnormalities could be a major disadvantage. In the present study, hydroxyproline in the urine of sheep on low and high planes of nutrition were measured before and after dosing with a slow-release formulation of dexamethasone. The object was to determine if a single injection of this formulation at the levels of dexamethasone which would cause defleecing resulted in a change in the hydroxyproline to creatinine ratio and hence in the normal rate of collagen and

possibly bone turnover. The possibility of altered bone turnover rates was further assessed by also measuring the urinary excretion rate of calcium. These effects of the steroid were compared with those obtained after giving sheep cyclophosphamide (CPA) which has also been studied as a defleecing agent (e.g. Gordon and Donnelly 1979) and which has been found to affect collagen metabolism in various rat tissues (Hansen and Lorenzen 1977). It was also of interest to determine the ratio of free to total hydroxyproline in the sheep and whether it was affected by the feeding level or drug treatment. In normal humans, this ratio is of the order of 5% and is only altered under certain abnormal conditions (Kivirikko 1970).

Materials and Methods

The Sheep, their Housing, Nutrition and Dosing

Two groups each of 10 adult Merino wethers were housed indoors in single pens and accustomed to a diet of lucerne chaff: oats (60:40) for a 6-week period prior to dosing with the defleecing agents. Five sheep in each group were offered 300 g/day (low plane) of the diet while the other five were offered 1200 g/day (high plane). Water was available *ad lib.* and the sheep were fed once daily at 0900 h. At dosing time, five of the low-plane and five of the high-plane sheep were injected intramuscularly with a dexamethasone formulation* at the rate of 1·15 mg dexamethasone per kilogram liveweight (*c.* 3 mg/kg$^{0.75}$) (for convenience these groups are referred to as the Dex-300 and Dex-1200 groups). The remaining 10 sheep were dosed orally with CPA (Gordon and Donnelly 1979) at the rate of 10 mg per kilogram live weight (CPA-300 and CPA-1200 groups). These treatments and mean liveweights of each group of sheep are summarized in the following tabulation:

Group name	No. of sheep	Mean liveweight (kg) ± s.e.m.	Feed intake (g/day)	Drug given
Dex-300	5	40·2 ± 1·8	300	Dexamethasone
CPA-300	5	40·7 ± 1·1	300	Cyclophosphamide
Dex-1200	5	51·5 ± 2·2	1200	Dexamethasone
CPA-1200	5	50·9 ± 1·5	1200	Cyclophosphamide

Urine Collection

Total urine collections were made by attaching a rubber sheath around the pizzle with Bostik (Bostik Australia, Pty Ltd) and allowing the urine to drain under gravity through this by means of plastic tubing attached to a nipple on the sheath. The tube passed through the grating of the floor into a collecting container below; this contained 100–200 ml of 6% (w/v) HCl as a preservative. Samples were collected over six periods, two of a week each, immediately before dosing, and four after; days 1 and 2 after dosing constituted the third and fourth periods, days 3–7 the fifth and days 8–14 the sixth. The acidified urine was stored at 4°C until analysed.

Blood Samples

Jugular blood samples were taken from all sheep by venipuncture into heparinized tubes, 5 days (day −5) prior to dosing and at 0900 h on day 0 prior to dosing at 1100 h. Post-dosing samples were taken after 3, 6, 24 and 27 h and then daily from days 2 to 14 and at day 23. After centrifuging at 6000 rpm for 15 min the plasma was drawn off and stored in capped vials at −4°C.

Assay of Urinary Hydroxyproline

Free hydroxyproline in the urine was measured by the automated method of Grant (1964). Total hydroxyproline was measured similarly after first autoclaving the urine (4 ml) with 10 M HCl (6 ml) in sealed tubes for 3 h at 120°C.

*Dexafort [Intervet (Aust.), Artarmon, N.S.W.] contains in 1 ml, 1 mg sodium dexamethasone-21-phosphate and 2 mg dexamethasone-21-phenylpropionate.

Assay of Urinary Calcium

Urinary calcium was assayed by atomic absorption spectrometry (Willis 1961).

Assay of Urinary Creatinine

Urinary creatinine was measured by the method of Owen *et al.* (1945) adapted for use with a Technicon autoanalyser.

Assay of Plasma Dexamethasone

The plasma samples taken from the sheep dosed with Dexafort were analysed in duplicate by the immunoassay method described by Panaretto (1979). The limit of sensitivity of the assay was 3·1 ng/ml and the mean coefficient of variation (± s.e.m.) based on the analysis of three standard curves was 4·9 ± 0·7%. Intrassay coefficients of variation in three assays varied from 3·8 to 5·6% in the concentration range 3·1–12 ng/ml. Quadruplicate analyses of the original Dexafort solution for dexamethasone by this method gave values which averaged 101 ± 4% (± s.e.m.) of the nominal concentration indicated on the label.

Results

Live Weights and Appetites of Sheep after Dosing

During the overall period of urine collection the average daily live weight changes (± s.e.m.) were −21 ± 8, −17 ± 10, +83 ± 5 and +117 ± 10 g for the Dex-300, CPA-300, Dex-1200 and CPA-1200 sheep, respectively. Most of the Dex-1200 sheep showed a depressed appetite after dosing but this was not observed in any of the other groups.

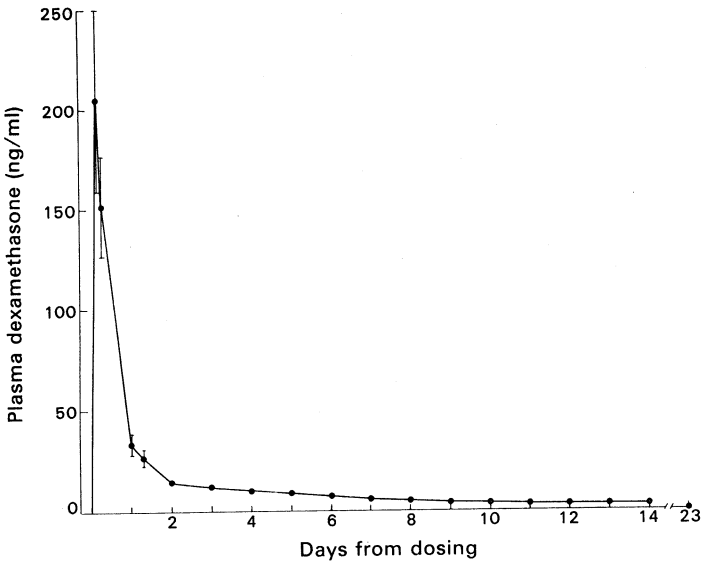

Fig. 1. Mean plasma concentrations (± standard error) of dexamethasone for 10 sheep, at various time intervals after giving a single intramuscular injection of a slow-release formulation of dexamethasone at day 0. (Five sheep were on a high plane and five sheep were on a low plane of nutrition.)

Plasma Levels of Dexamethasone after Dosing

The mean plasma concentrations, expressed as dexamethasone, for all 10 Dexafort-treated sheep are shown in Fig. 1. The mean levels in the low-plane group were

higher than in the high-plane group but the differences were not statistically significant. The concentrations fell rapidly from between 70 and 533 ng/ml to below 20 ng/ml in 9 out of the 10 sheep within 2 days of dosing; no dexamethasone was detectable after 9–14 days.

Daily Excretion of Creatinine in Urine

From the data of Langlands (1966) the daily rate of urinary creatinine excretion in wethers can be expected to range from 23 to 29 mg creatinine per kilogram live weight. There was no effect of either drug on the corresponding index value in the present experiment but the mean value (± s.e.m.) for the 10 high-plane sheep (26·5 ± 0·7 mg/kg) was higher ($P < 0·01$) than that for the 10 low-plane sheep (22·8 ± 0·7 mg/kg) when averaged over the six collection periods. It is possible that this difference could be attributed to greater error in the collection of urine from the low-plane sheep. For this reason and also because periods 3 and 4 represented collection periods of only 1 day it was decided to express all data for the excretion of hydroxyproline and calcium as a ratio to creatinine rather than as the total daily output. This approach is consistent with the approach of others (Kivirikko 1970).

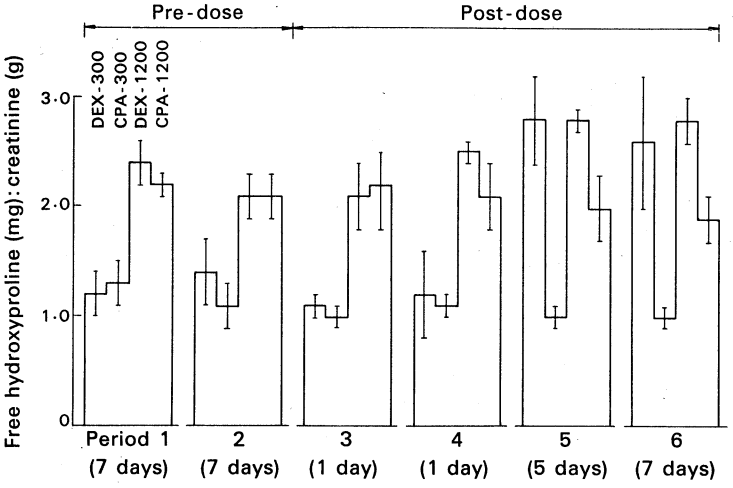

Fig. 2. Mean ratios (± standard error) of free hydroxyproline (mg) to creatinine (g) in urine from four groups of sheep (five per group) on different feed intakes (300 and 1200 g/day). The results refer to samples taken before (periods 1 and 2) and after (periods 3–6) dosing with either dexamethasone (Dex) or cyclophosphamide (CPA). Legend, see Materials and Methods. The treatment sequence indicated for period 1 is the same for all periods.

Ratio of Free Hydroxyproline (mg) to Creatinine (g) in Urine

Compared with the free hydroxyproline to creatinine ratios before dosing (periods 1 and 2) the values after dosing in periods 5 and 6 were higher in the Dex-300 (mean 111%) and Dex-1200 (26%) groups but there was little change in the CPA-treated sheep (Fig. 2). The ratios in the first 2 days after dosing (periods 3 and 4) were similar to those before dosing (periods 1 and 2) for all groups.

The 10 sheep on the high plane of nutrition excreted 76% more free hydroxyproline before dosing (2·20 ± 0·11 mg/g creatinine, i.e. mean ± s.e.m.; $n = 10$) than the 10 low-plane sheep (1·25 ± 0·11 mg/g; $n = 10$).

Ratio of Total Hydroxyproline (mg) to Creatinine (g) in Urine

Both groups treated with dexamethasone showed large increases in this ratio compared with pre-dose values but the effect was more pronounced in period 6 than period 5 and in the low-plane group compared with the high-plane group (Fig. 3). The ratios in period 6 were greater than the mean ratios for periods 1 and 2 by 268 and 84% for the Dex-300 and Dex-1200 groups respectively. With the CPA-1200 group there was a slight reduction in the ratio in periods 5 and 6 compared with the pre-dose values (Fig. 3).

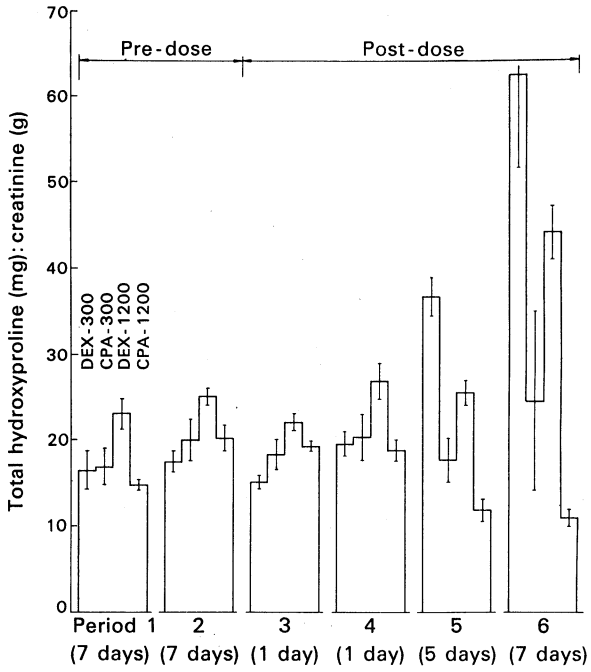

Fig. 3. Mean ratios (± standard error) of total hydroxyproline (mg) to creatinine (g) in urine from four groups of sheep (five per group) on different feed intakes (300 and 1200 g/day). The results refer to samples taken before (periods 1 and 2) and after (periods 3–6) dosing with either dexamethasone (Dex) or cyclophosphamide (CPA). Legend, see Materials and Methods. The treatment sequence indicated for period 1 is the same for all periods.

As observed for the free hydroxyproline to creatinine ratio the ratios for the total hydroxyproline to creatinine (periods 3 and 4) were similar to those in periods 1 and 2 (Fig. 3).

Before dosing (periods 1 and 2), in contrast to the values for free hydroxyproline the 10 high-plane sheep had mean values only 17% higher than the low-plane sheep; the respective mean values (± s.e.m.) were 20·70 ± 1·19 and 17·65 ± 1·10 mg/g creatinine. The Dex-1200 groups had fortuitously high values compared with the other three groups before dosing (Fig. 3).

Free Hydroxyproline as a Percentage of Total Hydroxyproline

Before dosing the free hydroxyproline represented $11 \cdot 0 \pm 0 \cdot 9\%$ (mean ± s.e.m.; $n = 10$) and $7 \cdot 4 \pm 0 \cdot 7\%$ of the total hydroxyproline in the high- and low-plane groups. These values tended to be slightly decreased by dexamethasone (to $4 \cdot 3 \pm 0 \cdot 5\%$) in period 6 for the low-plane sheep, due to a greater increase in the total than the free hydroxyproline, and increased by CPA (to $18 \cdot 9 \pm 3 \cdot 7\%$) in sheep on the high diet in periods 5 and 6, due to its inhibition of total hydroxyproline excretion.

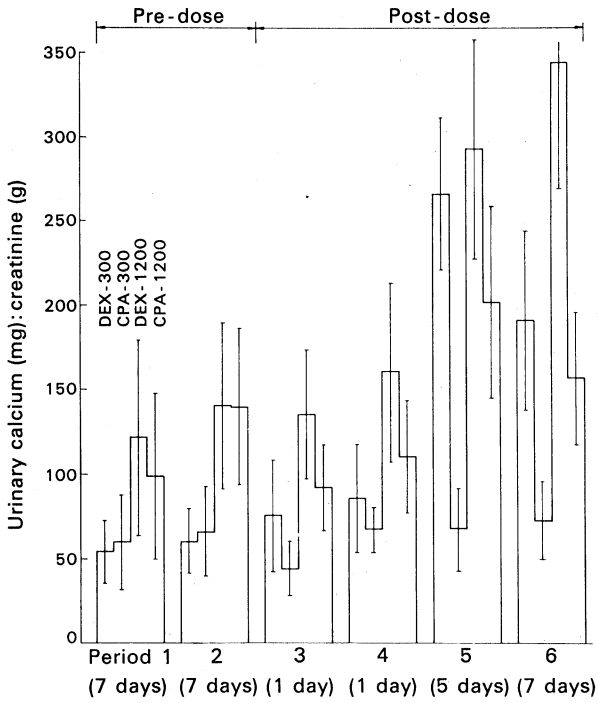

Fig. 4. Mean ratios (± standard error) of the total calcium (mg) to creatinine (g) in urine from four groups of sheep (five per group) on different feed intakes (300 and 1200 g/day). The results refer to samples taken before (periods 1 and 2) and after (periods 3–6) dosing with either dexamethasone (Dex) or cyclophosphamide (CPA). Legend, see Materials and Methods. The treatment sequence indicated for period 1 is the same for all periods.

Ratio of Urinary Calcium (mg) to Creatinine (g) in Urine

The ratio followed a pattern which was most similar to that for free hydroxyproline although the variability between sheep was much larger (Fig. 4). Compared with the pre-dose values (periods 1 and 2) the values after dosing in periods 5 and 6 were higher in the Dex-300 (mean 402%) and Dex-1200 (243%) groups. There was a tendency for the CPA-treated sheep to show higher values (42%) in these periods, but this was not as marked as seen after steroid treatment. The ratios in the first 2 days after dosing (periods 3 and 4) were similar to those before dosing (periods 1 and 2) for all groups.

The mean ($\pm$ s.e.m.) calcium excretion of the 10 high-plane sheep (125 $\pm$ 30 mg/g creatinine) was 108% greater than that for the low-plane group (60 $\pm$ 16 mg/g) in the two pre-dose collection periods, although the variability within the groups was so large that this difference was only statistically significant ($P < 0{\cdot}05$) in period 2 when tested using the *t*-test.

Urinary calcium excretion was more highly correlated with free than total hydroxyproline. When averaged over the six periods the respective correlation coefficients ($r \pm$ s.e.m.) were $0{\cdot}545 \pm 0{\cdot}057$ and $0{\cdot}431 \pm 0{\cdot}081$.

Discussion

Although hydroxyproline in urine is not derived exclusively from bone collagen the increase in both hydroxyproline and calcium in urine following dosing with the steroid, together with the known osteoporotic effects of steroid therapy in humans (Janoski *et al.* 1968), indicates that some bone resorption had taken place, particularly in those sheep in poor body condition. The correlation between urinary calcium and hydroxyproline excretion is consistent with the data of Klein *et al.* (1961). The best correlation was between free hydroxyproline and calcium excretion, suggesting that more of the free than total hydroxyproline in urine is derived from bone collagen, assuming that the calcium is derived from bone resorption. The duration of the effect cannot be determined from the present data but because the increase in the hydroxyproline to creatinine ratio had shown no signs of abating during the last measurement period the effect probably extended beyond the time during which urine was collected. Despite the use of a formulation which was designed by the manufacturers to release the dexamethasone slowly into the bloodstream over a period of 7–10 days, most of the steroid had disappeared by 2 days. It would be of interest to observe the effects of maintaining plasma levels of dexamethasone for longer periods at the higher levels found necessary for effective defleecing (Panaretto 1979). The doses of dexamethasone given in the present study were less than half those given by Panaretto (1979) and although large areas of wool were shed by these sheep the results were unsatisfactory from a defleecing viewpoint since wool could not be removed from some body regions.

Dexamethasone infusion was thought to decrease or even prevent collagen degradation in the skin of sheep (Leish and Panaretto 1979) so that although there was a thinning of the skin in the sheep in the present experiment (unpublished data) catabolism of skin collagen should not have contributed to the increased output of urinary hydroxyproline. However, it is quite likely that at least some other hydroxyproline-containing tissues (Robins 1977) have also been affected.

The marked increase in the hydroxyproline to creatinine ratio found in the sheep given dexamethasone is the opposite of findings by others (for review see Kivirikko 1970) in rats and humans. In these species administration of pharmacological doses of various glucocorticoids or ACTH markedly reduces the excretion of hydroxyproline in urine in the young and causes very slight decreases or no change in urinary hydroxyproline excretion in the adult (Kivirikko 1970). In Cushing's syndrome (adrenal cortical hyperfunction) in adult humans no change in urinary hydroxyproline excretion has been found (Kivirikko 1970) yet the incidence of osteroporosis in such patients ranges from 50 to 60% (Janoski *et al.* 1968). This suggests that the changes currently observed in hydroxyproline excretion by sheep could reflect skeletal abnormalities other than osteoporosis.

Expressing the results for hydroxyproline and calcium excretion as a ratio to creatinine rather than as total output per day had no effect on the conclusions drawn regarding the effects of the drugs. Because of the larger creatinine index found for the high-plane group the observed differences in the ratios between intake groups before dosing tended to be reduced using this mode of expression. Whether this difference in creatinine index indicates differences in the metabolism of creatine in the two groups, or is caused by errors in the collection procedure, remains to be determined.

In rats, semistarvation was found to cause a reduction in osteoid volume and in the number of osteoclasts (Shires *et al.* 1980). This was associated with a reduced hydroxyproline excretion and is consistent with the present results for sheep. However, these rats were hypercalciuric compared to their normally fed controls. In this experiment the underfed sheep were, by contrast, hypocalciuric compared to those on the high plane of nutrition.

The reason the high-plane sheep excreted relatively more free than total hydroxyproline compared with the low-plane sheep can be explained by several mechanisms: viz. (1) an increased proportion of collagen catabolized to the free amino acid; (2) a reduction in renal reabsorption of free hydroxyproline; (3) impaired oxidation of the free amino acid. Differences in these mechanisms would also account for the higher proportions of free hydroxyproline in sheep (*c.* 10%) compared to humans (*c.* 5%; Kivirikko 1970).

The tendency for CPA to suppress the total hydroxyproline to creatinine ratio in the CPA-1200 group indicates an inhibition of collagen synthesis in bone which may have been reflected in an increased calcium excretion in this group in periods 5 and 6. This is consistent with the observation that CPA, given daily for 2 weeks to rats, inhibited protein synthesis and proline hydroxylation in the skin, aorta and granulation tissue (Hansen and Lorenson 1977). The trend was not seen in the CPA-300 sheep because of lower rates of collagen synthesis in these sheep.

In conclusion, although these results confirm that skeletal problems could arise if corticosteroids are used as defleecing agents, whether this is a significant problem in practise would need to be assessed by long-term field trials.

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