Effect of ascorbic acid on urinary hydroxyproline of children receiving corticosteroids

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Liakakos, D., Ikkos, D. G., Vlachos, P., Ntalles, K., and Coulouris, Ch. (1974). *Archives of Disease in Childhood*, 49, 400. **Effect of ascorbic acid on urinary hydroxyproline of children receiving corticosteroids.** Oral administration of ascorbic acid, 0.5 g every 8 hours for a period of 4 days, had no effect on the urinary hydroxyproline excretion of 7 healthy children aged 8 to 14 years. When the same medication was given to 8 children aged 9 to 14 years who were receiving large doses of prednisolone (2 mg/kg per 24 hr) for a period of at least 15 days before as well as during ascorbic acid administration, a rise in the urinary hydroxyproline excretion was observed (from a mean value of 44 ± 4.1 SE before to 67 ± 4.6 mg/24 hr on the 4th day of ascorbic acid administration, $P < 0.001$). Urinary hydroxyproline excretion of 3 children 4 days after stopping ascorbic acid administration, while still on prednisolone, had returned to the level observed before ascorbic acid. It is concluded that large doses of ascorbic acid can, under acute conditions, neutralize the inhibitory action of corticosteroids on new collagen formation.

The adverse skeletal effects of long-term, continuous administration of pharmacological doses of corticoids—manifested by osteoporosis and in children by growth retardation as well—constitute one of the principal side-effects of corticoids that limit their therapeutic application (Travis and Sayers, 1967; Forsham, 1968; Rose, 1970).

Although the exact mechanism of the bone action of corticoids is unclear, it is known that corticoid administration is followed by a decrease in the formation of collagen (Siuko, Sävelä, and Kulonen, 1959; Kivirikko, 1963), and this is shown by a decrease in the urinary hydroxyproline excretion in children (Kibrick *et al.*, 1968; Liakakos *et al.*, 1971). The significance of ascorbic acid in the formation of collagen has been known for a long time (Wolbach and Howe, 1926; Hunt, 1941; Gould and Woessner, 1957) and more recently it has been shown that ascorbic acid can neutralize the inhibitory action of different substances, e.g. puromycin, on new collagen formation (Jeffrey and Martin, 1966a).

We here report a study of the effect of large doses of ascorbic acid on the inhibitory action of corticoids on new collagen formation in children.

Material and methods

The material of the present study consisted of two groups of children. Group A (control group) comprised 7 children (4 males and 3 females), aged 8 to 14 years, who were in hospital for minor ailments and were receiving no treatment. Group B (prednisolone group) comprised 4 males and 4 females, aged 9 to 14 years, of whom 6 had acute rheumatic fever and 2 had nephrosis (Table II). All children of group B were treated with prednisolone at a dose of 2 mg/kg per 24 hr for at least 15 days before the study, as well as during the present study.

All children of both groups received for 4 days ascorbic acid orally, at a dose of 0.5 g every 8 hours. Collections of 24-hour urine for measurements of hydroxyproline excretion were performed on the day before, and on the last (4th) day of, ascorbic acid administration. In 3 of the children in group B (Cases 8–10 in Table II) 24-hour urine samples were also collected on the 4th day after discontinuation of ascorbic acid administration.

On the day before and on the day of collection of urine samples a diet free of gelatin, meat, and fish was given to all children. The urines were collected under toluol and stored at -25°C until analysed for total hydroxyproline content by the method of Kivirikko, Laitinen, and Prockop (1967).

The statistical analysis of the results used standard techniques, as described by Trichopoulos (1971) and Snedecor and Cochran (1971).

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Results

The results of the present study are summarized in Tables I and II for groups A and B, respectively, in the Fig.

TABLE I

Urinary hydroxyproline excretion of healthy children (group A) before and during administration of ascorbic acid (0.5 g every 8 hours)

Case no.	Sex	Age (yr)	Urinary hydroxyproline (mg/24 hr)	
			Before*	During* (4th day)
1	F	8	44	39
2	F	9	41	22
3	F	10	48	50
4	M	11	50	52
5	M	11	51	46
6	M	13	66	70
7	M	14	92	101
Mean value			56	54
			6.7	9.5

*Before or during administration of ascorbic acid.

As shown in Table I ascorbic acid administration (0.5 g/kg 8-hourly for 4 days) to the children of the control group (group A) had no effect on the urinary hydroxyproline excretion, and the small changes observed (increase in 4 and decrease in 3) were not significant.

The same treatment resulted in an increase of the urinary hydroxyproline excretion of all children receiving prednisolone (group B, Table II), the difference of the values before and after ascorbic acid being highly significant by the paired *t*-test ($t = 5.919$, $P < 0.001$).

The mean urinary hydroxyproline excretion of the children of group B on the 4th day of ascorbic acid administration (67 ± 4.6 mg SE per 24 hr) was approximately 52% greater than before ascorbic acid (44 ± 4.1 mg SE per 24 hr) and did not differ significantly ($P > 0.05$) from the control value of group A (56 ± 6.7 mg SE per 24 hr).

The urinary hydroxyproline values on the 4th day of discontinuing ascorbic acid in Cases 8–10 of group B (Table II) were lower than during ascorbic acid, and were quite similar to the control values of the subjects in all 3 cases.

Discussion

Hydroxyproline, formed by hydroxylation of proline (Stetten and Schoenheimer, 1944; Stetten, 1950; Prockop, Peterkofsky, and Udenfriend, 1962)

is found exclusively in collagen (Neuman and Logan, 1950) and the amount of urinary hydroxyproline excretion, free and peptide bound, on a hydroxyproline-free diet constitutes a reliable index of collagen metabolism (Prockop and Kivirikko, 1967). For example, children (Ziff *et al.*, 1956; Anderson, Bannister, and Tomlinson, 1965) and young experimental animals (Martin, Mergenhagen, and Prockop, 1961; Kivirikko and Laitinen, 1965; Smith and Allison, 1965) excrete more hydroxyproline than adult subjects and grown-up animals, respectively. A relation between urinary hydroxyproline and growth rate has also been shown (Jasin *et al.*, 1962; Zorab *et al.*, 1970).

Administration of corticoids results in a decreased formation of collagen (Siuko *et al.*, 1959; Kivirikko, 1963), with a decreased urinary hydroxyproline excretion (Kibrick *et al.*, 1968; Liakakos *et al.*, 1971) associated with inhibition of growth in children (Van Metre and Pinkerton, 1959; Falliers *et al.*, 1963; Blodgett *et al.*, 1965).

Because of the well-known large variations of the urinary hydroxyproline values in children (Ziff *et al.*, 1956) and the small number of subjects studied by us, no statistically significant difference ($t = 1.55$, $P > 0.05$) could be found between the subjects of the two groups with respect to the urinary hydroxyproline values before ascorbic acid. As shown, however, in the Fig., subjects receiving prednisolone tended to have lower values than the control

FIG.—Urinary hydroxyproline excretion of healthy children (group A) and children on prednisolone for at least 15 days (2 mg/kg per 24 hr) (group B) before, during (4th day), and after (4th day) oral administration of ascorbic acid (0.5 g every 8 hours).

TABLE II

Urinary hydroxyproline excretion before, during, and after administration of ascorbic acid (0.5 g every 8 hours) to children treated with prednisolone (2 mg/kg per 24 hr) for at least 15 days before as well as during the present study (group B)

Case no.	Sex	Age (yr)	Diagnosis	Prednisolone (2 mg/kg per 24 hrs) for	Urinary hydroxyproline (mg/24 hr)		
					Before*	During* (4th dy)	After* (4th dy)
8	F	9	Rheumatic fever	18 dy	40	74	54
9	F	9	" "	18 "	34	38	32
10	M	10	Nephrosis	20 "	48	78	55
11	F	10	" "	20 "	51	76	
12	F	11	Rheumatic fever	20 "	22	60	
13	M	12	" "	15 "	57	74	
14	M	12	" "	15 "	54	68	
15	M	14	" "	15 "	41	67	
Mean value					44	67	(47)
SE					4.1	4.6	

*Before, during, or after administration of ascorbic acid.

subjects. Thus, values larger than 60 mg/24 hr were found in 2 of the 7 controls and in none of the 8 subjects of the prednisolone group.

The exact mechanism of the inhibitory action of corticoids on collagen formation has not been fully elucidated. The view that corticoids inhibit hydroxyproline synthesis, possibly during the hydroxylation of proline (Chvapil, 1959) is supported by the experimental results of Daughaday and Mariz (1962), who showed that addition of cortisol to cartilage culture *in vitro* inhibited hydroxylation of proline to hydroxyproline and, consequently, new collagen formation.

Although the specific role of ascorbic acid on collagen synthesis has been known for a long time (Wolbach and Howe, 1926; Hunt, 1941; Gould and Woessner, 1957), only in recent years has it been shown that its primary effect is on hydroxylation of proline after formation of protocollagen by peptide binding (Jeffrey and Martin, 1966a; Udenfriend, 1966; Comstock, Gribble, and Udenfriend, 1970) and that ascorbic acid acts as a co-factor of the specific enzyme (Hutton, Tappel, and Udenfriend, 1966; Juva and Prockop, 1966).

Jeffrey and Martin (1966b) studied the influence of ascorbic acid depletion on the growth of embryonic chick tibias *in vitro*. Of the parameters studied, the rate of collagen synthesis was particularly sensitive to ascorbic acid deficiency. After 4 days in culture without ascorbic acid, collagen synthesis virtually ceased. Addition of ascorbic acid to previously depleted tibias restored the rate of collagen synthesis to normal within 12 hours.

The same authors reported that puromycin

inhibited the synthesis of peptide-bound hydroxyproline. Simultaneous addition of ascorbic acid and puromycin to bones prelabelled with ^{14}C proline increased the amount of peptide-bound hydroxyproline. Peck, Birge, and Brandt (1967), working with cultures *in vitro* of isolated bone cells, arrived at similar results.

These facts, together with reports that ascorbic acid can hamper the inhibitory action of different substances on the hydroxylation of proline (Jeffrey and Martin, 1966a) led us to consider the possibility that large doses of ascorbic acid might eventually impede the inhibitory action of corticoids on new collagen formation.

Such a hypothesis is in keeping with the increased urinary hydroxyproline we observed after administration of ascorbic acid to children receiving large doses of prednisolone, from which it appeared that the inhibitory action of corticoids on new formation of collagen could be neutralized by large doses of ascorbic acid, at least under the conditions and for the duration of these studies.

That these results were not due to errors of methodology, i.e. interference of ascorbic acid in the urine on the determinations of urinary hydroxyproline, is proved by the fact that hydroxyproline excretion was unchanged during ascorbic acid administration to children not receiving prednisolone (Table I).

Although measurements of blood ascorbic acid levels were not performed, the possibility that patients treated with prednisolone were suffering from a subclinical deficiency of ascorbic acid, due to their illness and possible anorexia, and that the subsequent rise in total hydroxyproline after

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ascorbic acid was due to the treatment of this mild deficiency, seems highly improbable in view of the immediate drop of the urinary hydroxyproline values after discontinuation of the ascorbic acid, i.e. at a moment when subclinical ascorbic acid deficiency can be excluded.

It remains to be seen whether continuous administration of large doses of ascorbic acid during long-term therapy with corticoids will be able to prevent the skeletal side-effects of corticosteroid treatment.

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