Gingival epithelium in scorbutic guinea pigs

(Cavia procellus, Rodentia, Mammalia)

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SUMMARY

Guinea pigs submitted to an ascorbic acid deficient diet, but receiving as supplement, per os, a mixture of all other vitamins, could be kept alive, suffering from the vitamin C deficiencies for a longer period of time than those not supplemented.

The deficient animals, manifest alterations in the marginal gingival epithelium (external epithelium) and the attached gingiva, and in the vestibular areas of the molars of the upper and lower gingivae.

In these areas the epithelium manifest degenerative processes which show up as vacuoles in the cytoplasm of the cells of the basal and squamous cell layers (HE staining).

Keratin formation also occurs in the epithelial area, characterized by the appearance of unorganized and irregular keratin, represented by lamellae growing in various directions, and condensation zones, the presence of "shadow ghost cells" in the corneal layer was also noted.

INTRODUCTION

The first reference to hemorrhagic disease in Guinea pigs maintained on a cereal diet without "greens" is attributed to T. Smith (1895) (1). The signs and symptoms of scurvy in this animal were described later on by various authors (2, 3, 4), as being: loss of weight, sensitivity and swelling of the

1. Paper written by Rebeca de Angelis because of the death of the first authors in 1972.

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joints, and death on the 12th-14th day. Necropsy evidenced muscular hemorrhages, especially in the hind limbs near the joints, subcutaneous hemorrhages, and hemorrhages of the peritoneum and in the intestinal lumen, fragility of the bones, especially of the limbs, maxilla and mandibula, with marked looseness of the inferior molars, and hyperemia and volemia of the gingiva of the lower incisors.

Several authors (5, 6) did not find alterations of the gingival tissue, while others (7, 8), comparing the cicatrization in normal or scurvy Guinea pigs, observed the increase of mucopolysaccharides in the conjunctiva of the scorbutic animals; Robertson and Hinds (9) observed an increase in hyaluronic acid in the conjunctiva, under the same conditions.

More recently, it was observed (10, 11), inflamation, edema, necrosis, hemorrhages, and fragmentation of the gingival chorionic collagen in scorbutic monkeys and, with reference to the gingiva, they found necrosis and leukocytic infiltration on the area of the gingival crevice. It was (12) observed alterations of the epithelial tissue of the oral cavity in Guinea pigs with Vitamin C deficiency, however, found them to be secondary to the alterations of the gingival epithelium.

The present paper reports a study of the vestibular gingiva of the molars, i. e., the external epithelium of the marginal and attached gingiva in normal and Ascorbic acid deficient Guinea pigs. It was the ambiguity found in the literature about this subject what prompted us to undertake this work.

MATERIAL AND METHODS

Male guinea pigs (Cavia porcellus) were used through out this work. Two experiments were carried out:

Experiment 1

Fourty six guinea pigs with an initial weight of 210-365 g ($X = 290.5 \pm 37.0$ g) were kept for 24 days on a colony diet supplemented with cabbage (approximately 13g/animal/day) in order to have similar nutrition conditions at the beginning of the experiment (Adaptation diet). The mean final weight at this stage was 345.5 ± 27.2 g.

This period was followed by a diet deficient in ascorbic acid (Table I, Diet A) till death occurred (mean 25 days). Eight animals received a supplement of ascorbic acid (10 mg/day) and were the control group, and were observed for 40-50 days.

Experiment 2

Thirty two guinea pigs with an initial weight of 175-605 g ($X = 387.5 \pm 50.0$) were kept on the adaptation diet to the laboratory conditions, as in Exp. 1, for 8 days. The final mean weight at this period was 393 ± 48g; this period was followed by a vitamin C deficient diet, supplemented with an "extra" oral vitamin mixture (Table II) an alternate days. The observations were for 45 days.

Five of these Guinea pigs received a supplement of 30 mg/day of Vitamin C by the oral route after 30 days on the Vitamin C deficient diet, and after 30 days, this supplementation was again discontinued.

The "Control" group received since the beginning 10 mg/day of ascorbic acid by oral route (12 animals).

The composition of the diets and supplements are reported in Tables I and II.

METHODS

The animals were weighed every other day.

The ascorbic acid was determined in the liver and in the experimental diets using the 2,4-dinitrophenylhydrazine method (13, 14).

Histological technics - immediately after the animals were sacrificed, the gingiva of the vestibular area of the molars was removed with scalpel and scissors. The tissue was immersed in Gendre's solution previously chilled and kept at 4°C for 24 hours (lower gingiva) or in Bouin's solution (upper gingiva).

After inclusion in parafin, 5 u slices were obtained and stained with HE according to Malory and van Gieson. Polarized light was used for the examination.

TABLE I
COMPOSITION OF EXPERIMENTAL DIETS (%)

	EXP. 1(a)	EXP. 2(b)
Corn meal	—	20
Whole oats ground	52	40
Whole wheat ground	20	23
Meat meal	—	5
Casein	12	5
Ground cellophane paper	5	—
Cotton seed oil	4	2
Corn oil	4	2
Mineral supplement*	3	3
Vitamin A	10.000 IU	10.000 IU
Vitamin D	500 IU	500 IU
Vitamin supplement**	**	**

* Calcium glycerophosphate, magnesium glycerophosphate, iodinated sodium chloride (equal parts).

** Thiamine HCl, 6 mg; Riboflavin, 16 mg; pyridoxine, 16 mg; calcium pantothenate, 20 mg; nicotinic acid, 30 mg; folic acid, 5 mg; vitamin B_{12} , 5 mg; p-aminobenzoic acid, 100 mg; 2-methyl-1,4-naphthaquinone, 50 mg; inositol, 2g; choline HCl, 1.5g; vitamin E, 52 mg. (Per Kg/diet).

(a) The ascorbic acid content of this diet was 2.5 mg/100g. (Almost 1/4 of the requirement (21), since the mean ingested was 0.75 mg of vitamin C/animal instead of the recommended 3 mg/animal).

(b) The ascorbic acid content of this diet was 1.18 mg/100 g (Almost 1/9 of the requirement (21), since the mean ingested was 0.32 mg of ascorbic acid/animal, instead of 3 mg/animal).

TABLE II
COMPOSITION OF THE "EXTRA" VITAMIN SUPPLEMENT GIVEN BY MOUTH (EXP. 2)

Vitamin	Quantity administered every other day	Estimated requirement animal/day
Thiamine HCl	0.4 mg	0.2 mg
Riboflavin	0.8 mg	0.4 mg
Pyridoxine	0.8 mg	0.4 mg
Calcium pantothenate	1.0 mg	0.5 mg
Nicotinic acid	2.0 mg	1.0 mg
Folic acid	0.2 mg	0.1 mg
Vitamin B_{12}	0.4 mg	0.2 mg
p-Aminobenzoic acid	4.0 mg	2.0 mg
2-Methyl-1,4-naphthaquinone	3.0 mg	1.5 mg
Inositol	120.0 mg	60.0 mg
Choline HCl	90.0 mg	45.0 mg
Vitamin E	3.2 mg	1.6 mg

RESULTS

Ascorbic acid in the liver:

The liver ascorbic acid content in the 1g Experiment was: 0.28 mg/100 g and 8.0 mg/100 g of wet weight for the deficient and controls animals respectively.

In the second experiment, the liver ascorbic acid content was: 0.29 mg/100 and 18.1 mg/100 g wet weight for deficient and controls animals respectively.

Weight:

The behavior of the weights (mean) for the 1º and 2º Experiments are found in Fig. 1 (a and b).

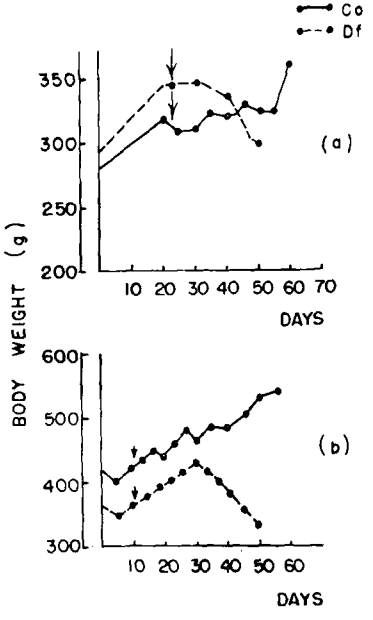

Fig. 1. Variations in body weight of the guinea pigs receiving the diet supplemented with ascorbic acid (Co) and withouth this supplementation (Df)
a – Experiment 1
b – Experiment 2

The animals of the 2nd group, receiving after the 39th day a supplement of vitamin C to the 60th day, responded by weight increase, while they lost weight abruptly when the supplement of vitamin C was discontinued (Fig. 2).

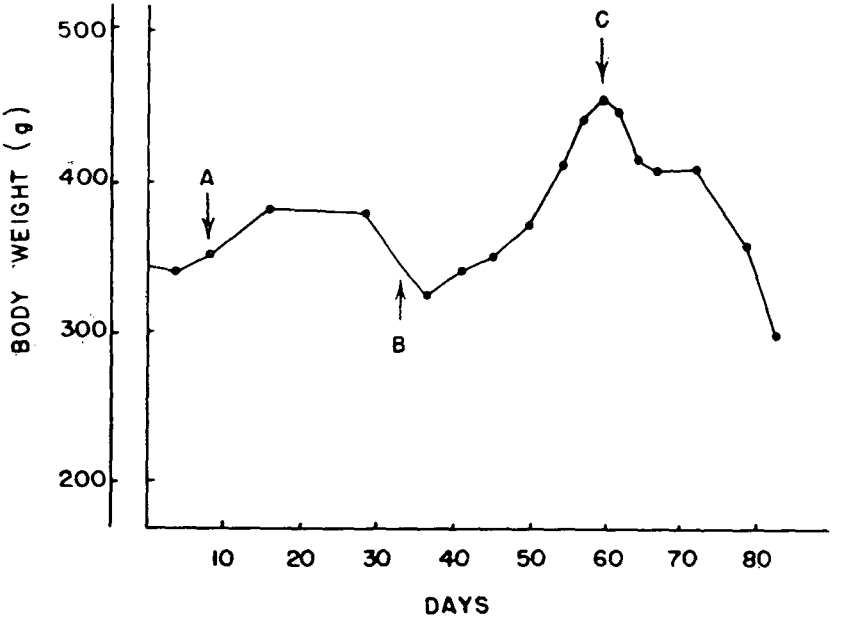

Fig. 2. Variation in body weight of the guinea pigs on the diet without ascorbic acid (A to B), receiving from the 30th day on an ascorbic acid supplementation (30 mg/day/animal). (B to C), which was withdrawn at point C.

Mathematical analysis

2nd Experiment, weights - starting from the hypothesis that the evolution of the weights along the whole test follows a 2nd degree equation, we deduce the values of the coefficients a, b and c: $Ey_i = aEx_i^2 + bEx_i + nC$, where y_i is the weight of the animals on day x_i and this was followed for the weightings during the ascorbic acid deficiency period (n = number of observations).

From

$$Ey_i = aEx_i^2 + bEx_i + nC$$

$$Ey_i x = aEx_i^3 + bEx_i^2 + cEx_i$$

$$Ey_i x^2 = aEx_i^4 + bEx_i^3 + cEx_i^2$$

We obtain:

$a = -0.129$

$b = 5.5$

$c = 345$ and the following equation results:

$$y = -0.129x^2 + 5.5x + 345$$

Solving the equation for $dy = 0$, we get for the maximum

$$x = 21.318$$

Comparing the value obtained for y by the equation with the values observed, a correlation is noted (Table III).

TABLE III

WEIGHTS (MEAN) OBSERVED ON DAYS (x) COMPARED TO THE WEIGHTS CALCULATED BY THE EQUATION $Yi = -0.129x^2i + 5.5xi + 345$, WHERE γi = WEIGHT OF THE ANIMAL ON DAY xi

x	Weigth observed	Weigth calculated
1	357.0	350.37
5	367.5	375.72
10	386.0	387.10
15	393.5	399.47
21	395.7	403.61
25	391.0	401.90
30	374.5	394.00

Chorion

The examination of the gingival chorion showed the following alterations in comparison with the controls:

a — edema, dissociation of the collagen bundles, and fragmentation of the collagen fibers.

b — increase in the number of lymphocytes, presence of neutrophyles, sometimes of eosinophyles, and hemorrhages and fibrinoid necrosis.

c — infiltration of numerous cells which, according to the roundish nuclei, loose chromatin and hardly visible cytoplasm, could be considered undifferentiated mesenchymal cells.

d — capilary neoformations, cappilary and venous congestion and swelling of the endotheliocytes.

Epithelium

The examination of the gingival epithelium, in comparison with the controls, showed the following alterations:

In the squamous cell layer, cells with light citoplasm or small cytoplasmatic vacuoles; in the basal layer, frequent cells with a very evident membrane, central nuclei or semilunar nuclei at the periphery, and colorless cytoplasm. This suggests hydropexic degeneration. More frequent mitosis in the basal layer. Apparent hyperplasia of the squamous cell layer and absence of in tapapillary cones and dermic papillae with a subsequent increase in the thickness of the epithelium. In one of the animals almost all cells of the squamous cell layer were binucleated.

Neutrophyles infiltrating the epitheium and intraepithelial hemorrhages were observed with hematocytes between the squamous cells and granular layers or between the granular and corneal layers. Necrotic foci of intraepithelial coagulation.

Among the epithelial alterations the most frequent were those of the keratin. At times, the keratin would loose its structure of parallel lamellae, acquiring an irregular disposition with lamellae in different directions, an ateration named by us "irregular keratin", and, sometimes, condensation of keratin inside the corneal layer separated by clear spaces (vacuoles).

The presence of irregular polyhedral formations of well defined outline was noted with a homogenous and paler interior, somewhat like the cells of the squamous cell layer. These we named "shadow ghost cells" in the keratin.

In some animals "corneal pearls" were observed and in one animal, one cell of the interior of the granulosa was in a state of precocious keratinization.

The microphotographs in Figs. 4 to 7 show some of these alterations in comparison with the normal controls. In Fig. 3 we find alterations of the lower gingiva.

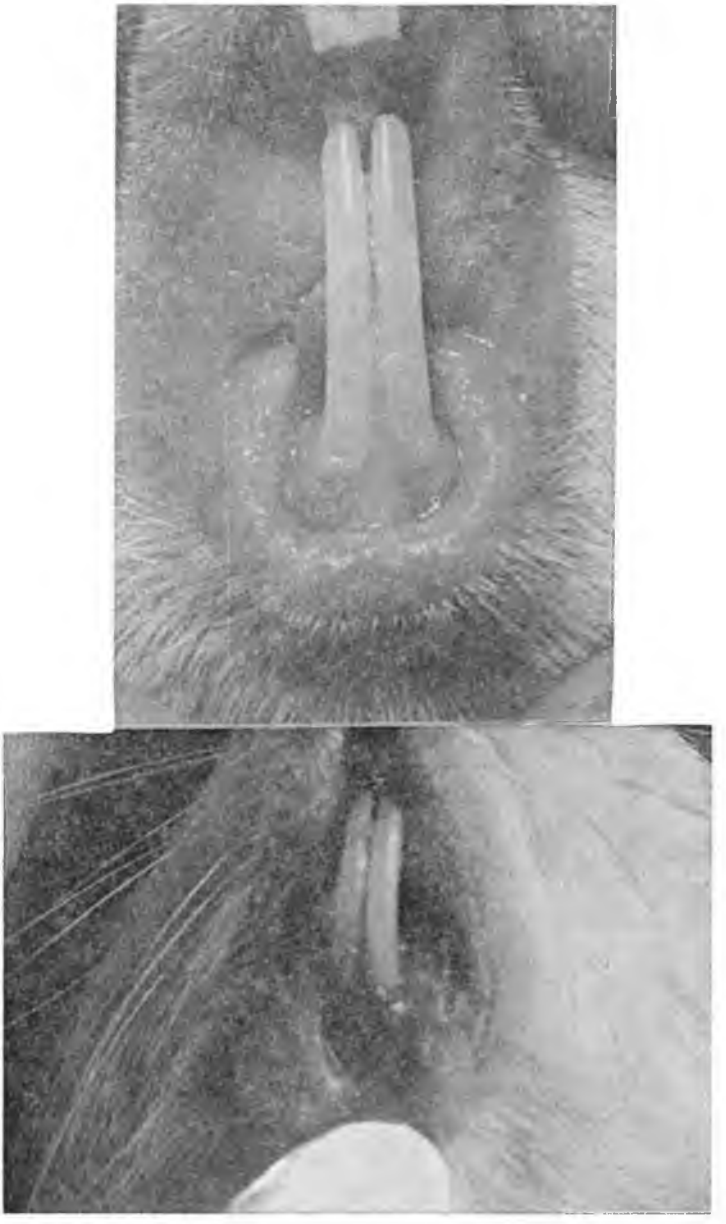

Fig. 3. Top - Guinea pig receiving Vitamin C (Co)
Botton - Scurvy Guinea pig (Df).

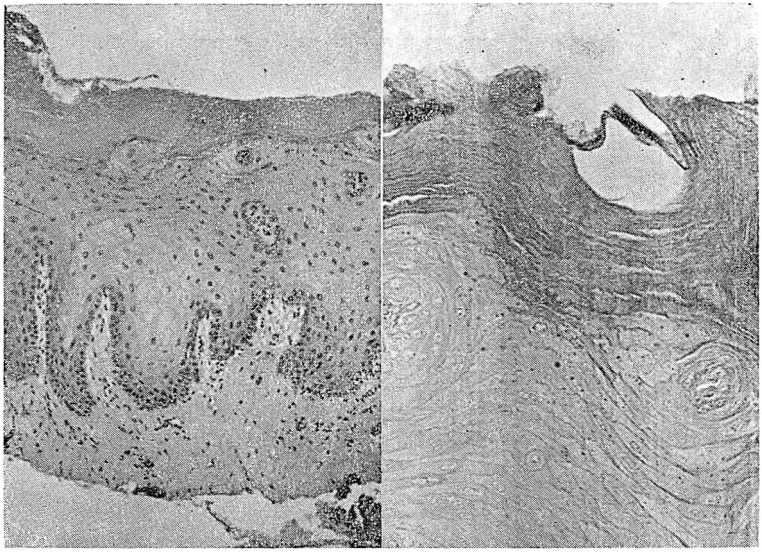

Fig. 4. Sections of the gingiva of the Co guinea pig (Exp. 2). In the epithelial tissue: in the basal and squamous cell layers, irregular polyhedral cells; granulosa with keratin-hyaline granules; cornea with keratin lamellae. To the right: positive reaction of the corneal layer (reaction of Chévremont and Frédéric) (120x) (20).

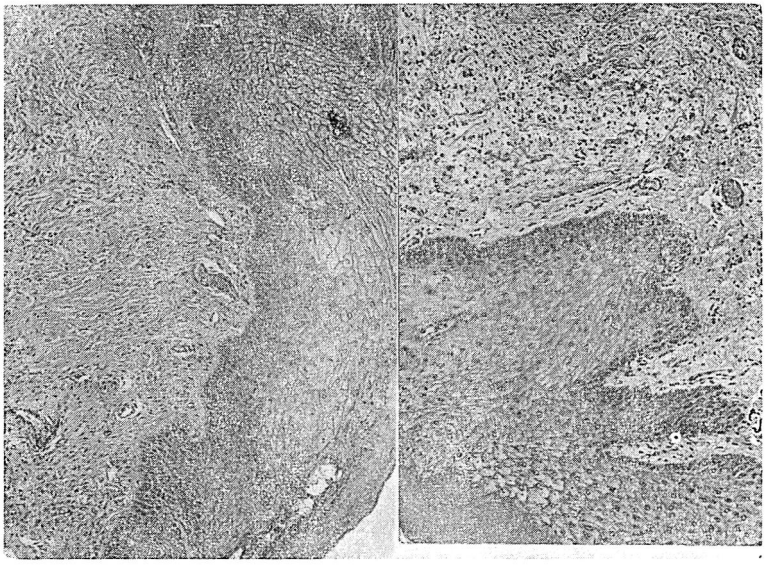

Fig. 5. Intraepithelial hemorrhage, vascular congestion, edema of the chorion. Infiltration of 1 lymphocytes and some leukocytes (HE, 120x).

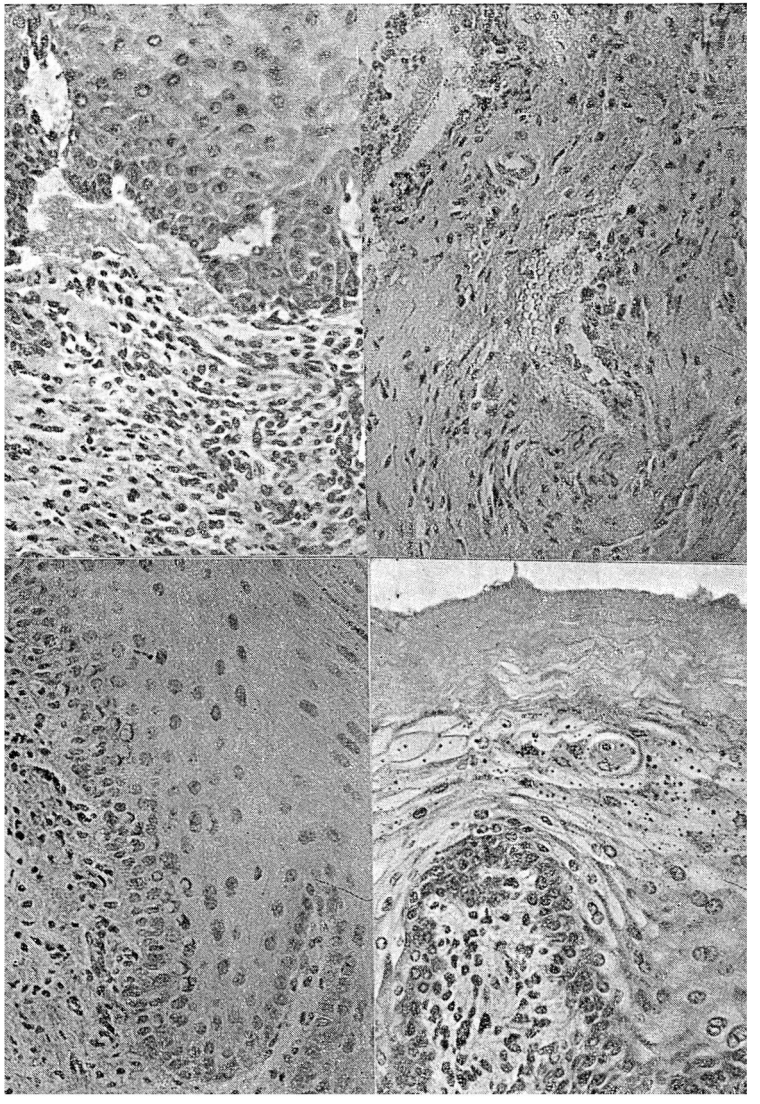

Fig. 6. Focus of fibrinoid necrosis with infiltration of immature cells in the chorion. In the 2nd photograph, swelling of endotheliocytes. In the 3rd photograph, cells of the basal layer with a semilunar nucleus at the periphery. In the last photograph, premature keratinization with irregular keratin to the right and normal keratin to the left (HE, 300x).

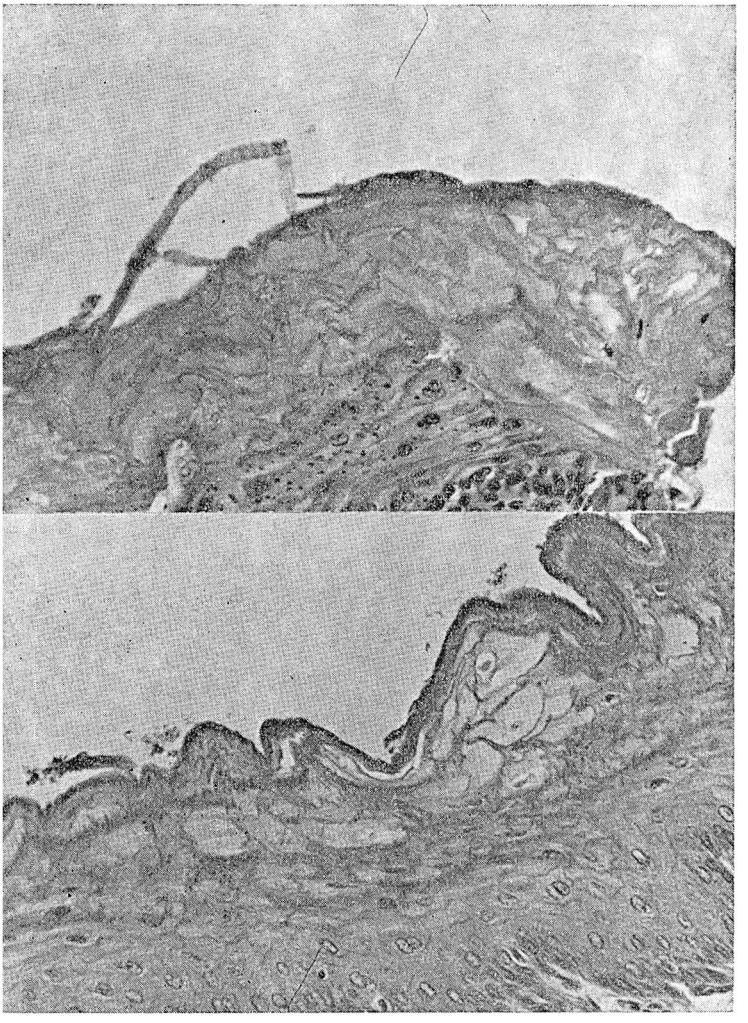

Fig. 7. In the top photograph, irregular keratin "vacuoles", and "shadow ghost cells" in the keratin. In the bottom photograph, degeneration of the squamous cell and granulous layers. "Abnormal" keratin. (HE, 750x).

DISCUSSION

Weight

The diets (Exp. 1 and 2) the animals were fed for vitamin C deficiency were realy low in their ascorbic acid content (Table. 1), and as they were supplemented with all other vitamins, the observed alterations must be due to vitamin C deficiency, and not to other vitamin.

Since the supplements of ascorbic acid given to a group of animals inhibited the appearance of all alterations noted, it is strongly suggested that these alterations were due to the deficiency in ascorbic acid.

During the ascorbic acid deficiency period the guinea pigs went through a latency period during which they continued to grow, up to a certain moment constant for all animals observed, followed by rapid weight loss. This behavior was interpreted mathematically:

From the analysis carried out we have:

$y = -0.129x^2 + 5.5x + 345$ with an inflexion point for

$x = 21.318$ (days).

The ageement between the calculated data and the results obtained in the experiment suggests that under the conditions used in experiment 2, after the onset of vitamin C depletion, the animals continue to grow up to the 21st or 22nd day, starting to loose weight after this latency period.

Other experiments carried out in our laboratory under the same conditions, produced the same behavior pattern (unpublished observations).

Histology

As to the macroscopic alterations of the gingivae (Fig. 1), subcutaneous and muscular hemorrhages and hemorrhages in the lumen and wall of the digestive tracts, the mobility of the lower molars, the sensitivity of the joints when manipulated and the swelling of the tibio-tarsal joints, agree with the observations of Holst and Frölich, Cohen and Mendel, Sherman et al., and Hood et al. (2, 3, 4, 15, 16).

The very low levels of ascorbic acid in the liver show that the reserves of this vitamin were minimal.

The alterations of the gingival chorion coincide with those described for vitamin C deficient guinea pigs and monkeys by Glickman (1948), Turesky and Glickman (1954), Tomlinson (1942), and Waherhang (1958) (17, 18, 19, 10).

Since the overall picture is characteristic for vitamin C deficiency, it is reasonable to admit that the alterations observed in the gingival epithelium are also due to ascorbic acid deficiency. Waherhang (10) explains that the alteration of the gingival crevice (increased thickness of the epithelium) and the presence of subgingival bacteria plaque are due to the destruction of the subjacent tissues, a hypothesis which can also be applied to explain the epithelial alterations found in our case.

The alterations described by us were found in the marginal gingiva (external epithelium) and the attached gingiva, excluding, thus, the possibility of they being due to an irritation produced by "plaques" or, eventually, salivary calculi.

These alterations consist primarilly of vacuolization of the cells of the basal epithelial layer, probably increased mitosis in the same layer, and alterations of the corneal layer, characterized by the formation of abnormal keratin with irregularities, vacuoles, and "shadow ghost cells".

The epithelial alterations were more accentuated during the 2nd experiment (duration 43 days) than during the 1st experiment (26 days), suggestion that a relatively prolonged period of deficiency is necessary to make them evident.

SUMÁRIO

Alterações do epitelio gengival de cobajos em carência de ácido ascórbico

Cobaios foram submetidos a um período de carência de ácido ascórbico, durante o qual receberam por via oral, suplemento vitamínico a fim de conseguir manter o período de deficiência por maior tempo sem morte do animal.

Nos animais carentes ocorrem alterações no epitélio gengival marginal (vertente livre) e inserida, na regiao vestibular dos dentes molares, tanto na gengiva superior como na inferior.

Nestas áreas, o epitelio apresenta processos degenerativos que, nas colorações de HE aparecem como vacúolos no citoplasma de células das camadas basal e espinhosa.

Ocorre também formação de queratina nestas áreas epiteliais, caracterizada por aparecimiento de queratina desorganizada, irregular, representada por lamelas em varias direções e zonas de condensação, havendo ainda "silhuetas de células" na camada córnea.

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