SURGERY
GYNECOLOGY AND OBSTETRICS
An International Magazine, Published Monthly

VOLUME 75 JULY, 1942 NUMBER 1

THE RELATIONSHIP OF THE CONCENTRATION OF ASCORBIC ACID OF THE BLOOD TO THE TENSILE STRENGTH OF WOUNDS IN ANIMALS

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THE healing of wounds is a complex phenomenon and cannot be described in terms of a few specific governing factors. The regulation and control of growth of tissue presents problems not understood, and since the healing of wounds is dependent on the growth of tissue the problem of healing is not easy. However, there is certain information available regarding deterrents to healing in surgical wounds in patients. Deterring factors may be classified as (1) general or (2) local. These have been discussed in recent reviews (5, 6, 10, 15).

From the Department of Surgery, Wayne University College of Medicine. This work was supported by grants from Frederick Stearns & Company and T. D. Buhl.

Read before the Forum on Fundamental Surgical Problems at the Clinical Congress of the American College of Surgeons, Boston, November 5, 1941.

In order that a wound may heal it must be at sufficient rest for the parts to adhere, but many mechanical factors found in surgical patients cause motion of the approximating edges. Imperfect suturing, activity on the part of the patient, coughing, straining, or rough handling on the part of attendants disturb the quiet of the wound. Locally, excessive trauma, infection, strangulation of tissue, presence of foreign bodies, and excessive tension, are factors which predispose to poor wound healing. These have been mentioned in most discussions of the subject; that they may cause difficulty in wound healing is obvious. Other factors, especially in clean and otherwise uncomplicated cases, are usually less apparent and must be considered as due to general disturbances in the patient, since

Fig. 3. Four day wound of a normal guinea pig. Note absence of exudate and partial healing of corium. Tensile strength 183 grams per centimeter of wound. Hematoxylin and eosin. ×20.

Fig. 4. Four day wound of a deficient guinea pig. Note exudate in corium and subcutis. Tensile strength 138 grams per centimeter of wound. Hematoxylin and eosin. ×20.

Fig. 5. Six day wound of a normal guinea pig. Note collagen production throughout wound. The tensile strength of this wound was 674 grams per centimeter. Azocarmine. ×20.

Fig. 6. Six day wound of a deficient guinea pig. Note pool of exudate in muscularis. No evidence of collagen in corium. The tensile strength was 291 grams per centimeter. Azocarmine. ×20.

Fig. 7. Eight day wound of a normal guinea pig. Note abundant collagen present throughout wound. The tensile strength was 1279 grams per centimeter. Azocarmine. ×20.

Fig. 8. Eight day wound of a deficient guinea pig. Note only small amount of collagen present. The tensile strength was 345 grams per centimeter. Azocarmine. ×20.

no local or mechanical cause is known. Concurrent disease and malnutrition probably are the most important of the general causes of poor wound healing. These have until recent years received little attention.

Experimental data have shown that vitamin C deficiency plays an important part in wound healing in the experimental animal. Aschoff and Koch in 1919 stated that in scurvy the main primary deficiency is a lack of intracellular cement substance. Hojer in 1924 verified these observations in the scorbutic guinea pig and noted changes which were due to a lack of collagen. Wolbach and Howes demonstrated that the histological basis for the failure of wounds to heal in the presence of vitamin C deficiency lies in the inability of the supporting tissues to produce and maintain intracellular substance, and later Menkin, Wolbach, and Menkin found that microscopic sections of tissues of scorbutic animals demonstrated the deposition of increasing amounts of intracellular cement substance depending on the amount of vitamin C administered. Taffel and Harvey reported that the tensile strength in stomach wounds of the partially scorbutic guinea pig is much decreased from the 8th to the 10th day. Lanman and Ingalls have shown that guinea pigs maintained on approximately one-fifth of the minimal protective dose of ascorbic acid exhibited healing of operative wounds inferior to those of normal controls. Von Jeney and Törö were able to demonstrate in *in vitro* cultures of fibroblasts a sudden increase in collagen fibrils when ascorbic acid was added to the medium. Wolbach (23) states that deficiencies of vitamin C result in abnormalities of all intracellular substances having collagen as their basis, and that its absence prevents the formation of the matrices of white fibrous tissue, bone, cartilage, and dentin. He states further that in vitamin C deficiency the pathological picture is produced by resorption of intracellular materials in both growth and reparative reactions. In such instances, if vitamin C is supplied either in natural foods or as the pure compound, histological repair is immediately resumed. Some of these findings were confirmed by Mazoue. Lauber and Rosenfeld in 1938 made histological studies of the vitamin C content of various tissues in the bodies of guinea pigs on low vitamin diets. When wound healing was in progress a decreased amount of vitamin C was found in the various tissues, suggesting that it had been utilized in the healing process. In a recent experimental study, Hunt found that collagen does not form in healing wounds of guinea pigs partially deficient in vitamin C, and that the intracellular substance remains immature and of poor holding power. He concluded that vitamin C in good concentration is of the greatest importance in wound healing.

It would seem that there is ample proof, many times confirmed, that vitamin C is necessary for the formation of collagen in tissue repair, and further that the development of tensile strength in wounds is retarded by a partial or complete vitamin C deficiency.

This report is concerned with a careful biochemical and histological study of wound healing associated with deficient vitamin C intake but with other factors maintained as constant as possible.

METHODS

Young guinea pigs were used, weight 300 to 450 grams at start of the experiment. Both males and females were included, being distributed evenly in control and vitamin C deficient groups. The basal scorbutogenic diet used in all experiments was arrived at by combining features of diets used by several previous investigators (13, 19, 21). Its composition is as follows: rolled oats, 40 per cent; skimmed milk powder, heated, 30 per cent; wheaten bran, 18 per cent; butter fat, 10 per cent; sodium chloride, 1 per cent; cod liver oil, 1 per cent.

The animals were kept on this diet plus an adequate oral ascorbic acid intake of 5 milligrams a day for at least a week before the start of the experiment. The controls were continued on this regimen throughout, while the vitamin C deficient animals were given no ascorbic acid for one week and 0.2 milligram per day thereafter. (Note: 2 mgm. per day is the threshold for tooth protection, according to Harris and Ray.) The average of the weight curves was approximately the same for

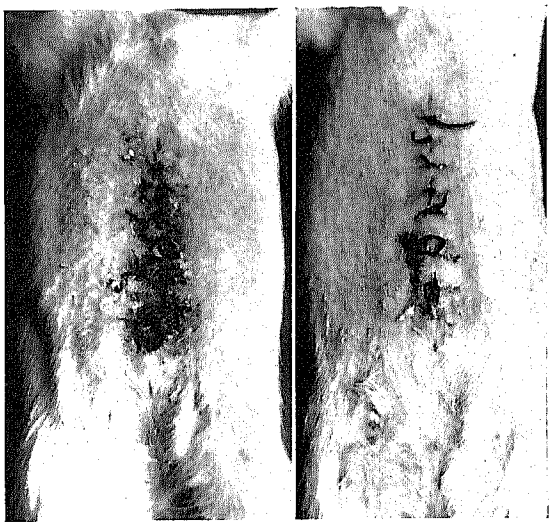

Fig. 1. Abdominal wounds in deficient and control guinea pigs on the 8th postoperative day. There is marked edema about the wound of the deficient guinea pig with heavy crusts still present. This presents a marked contrast to the wound in the control animal which appears quite healed.

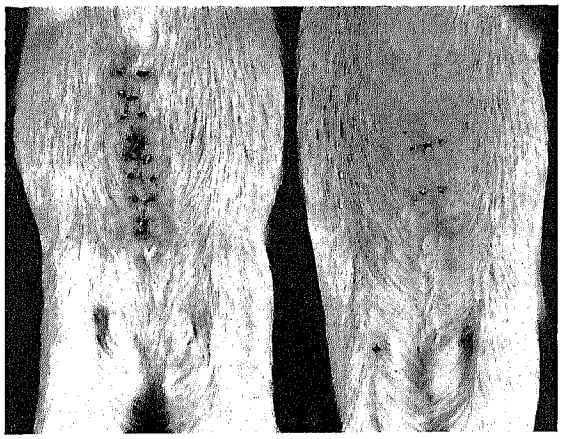

Fig. 2. Abdominal wounds in deficient and control guinea pigs on the 14th postoperative day. The stitches had been removed 5 days previously. There still are a few crusts present on the wound of the deficient animal and some thickening remains, while the wound of the control pig is soft, smooth and well healed.

the deficient and the control guinea pigs. Both groups gained until the day of the operation. They then showed a loss the day after operation. The downward trend was reversed from the 4th to the 6th day, after which they then continued to gain at about the same rate throughout the duration of the experiment.

The animals were operated upon 14 to 15 days after the start of the experiments. Midline abdominal incisions about 6 centimeters in length were made and sutured with silk. Nembutal anesthesia was used. The animals were sacrificed 4, 6, 8, 10, or 14 days later. Blood was obtained at this time for determination of plasma ascorbic acid and total protein. The blood was drawn from 5 to 10 hours after the last vitamin C was given. The plasma vitamin C value was determined by the method of Bessey, slightly modified. The plasma protein value was determined by the total nitrogen determination method of Pregl.

For the determination of wound strength, the entire belly wall was removed and the sutures all cut. Strips 1 centimeter in width (measured *in situ*) were cut perpendicular to the incision, by means of a sharp razor blade. The tensiometer was used to measure the grams per centimeter length of wound, as read directly from the tensiometer scale. Four or five such strips were obtained from each animal and the average calculated. Thus the average strength over the whole length of the wound may be estimated. In this respect the method differs from that used by others who measured the pressure required to burst the wound open, thereby obtaining the strength at the weakest point. For the purpose of conducting the histological studies a 5 millimeter strip was taken from near the center of the wound.

The piece of excised wound for histological study was fixed in 10 per cent formalin and paraffin sections were prepared. Routine hematoxylin and eosin and Heidenhain's azocarmine were stains used in the preparations of slides. The last mentioned stain was of some value in comparing the amount of collagen present in the wounds, but most of the changes were present with the routine hematoxylin and eosin stains. In comparing the controls and the test animals we found great variations in healing of wounds which could be ascribed to inaccurate approximation; therefore, comparisons were made of representative guinea pigs in the control group and representative guinea pigs in the deficient group.

RESULTS

There was a marked difference in the gross appearance of the wounds in those guinea pigs kept on diets rich in vitamin C and in those kept on a partially vitamin C deficient diet. This is in confirmation of Hunt's previous findings.

In the normal guinea pigs, healing progressed rapidly. The wounds were clean, there was no oozing of blood from the margins or about the sutures, and there was no edema about the wound. On section there was no thickening of the belly wall and no evidence of hemorrhage into the wound. They almost universally appeared well healed by the 6th to 8th postoperative day, and by the 10th to the 14th postoperative day the incision was white and smooth. (Figs. 1 and 2.)

In those animals fed vitamin C deficient diets, the wounds presented a very different appearance. Almost without exception there was edema along the suture lines with some redness. Heavy bloody crusts formed soon after operation and were almost invariably still present on the 8th to the 10th postoperative day. Although appearing well healed on the 14th day, the incision was still hard and raised, with some crusts still present. When the belly wall was sectioned it was found to be thickened and often presented a hemorrhagic appearance.

Microscopic study of sections of wounds in control and in partially vitamin C deficient animals were made and we are indebted to Dr. Mark E. Mann of the department of pathology for his aid in interpreting the histological data. This study revealed marked differences as early as the 4th postoperative day. In the control animal (see photomicrograph, Fig. 3, frontispiece) epithelial bridges were complete though the thickness of the epithelium varied in individual pigs. A slight exudation of serum, red blood cells, and neutrophils was found in the corium and between the muscle bundles. A moderate fibroblastic proliferation was evident in the deep portion of the corium, but only a few thin collagenous fibers could be seen. Numerous foreign body giant cells and focal collections of mononuclears were detected at the edges of the wounds. Examination of the slides of the 4 day deficient group (Fig. 4, frontispiece) revealed the epithelium to be well healed. In the reticular portion of the corium and in the subcutis numerous pools of serum containing red blood cells and a few lymphocytes could be found. Fibroblastic proliferation was more marked than in the control animals, but collagen production was scant. The presence of a hemorrhagic effusion was constant in all deficient animals.

At 6 days the wounds of control animals (Fig. 5, frontispiece) indicated progressive healing by an increase in fibroblastic proliferation; the production of some mature collagen and of numerous thin blue-staining fibers was noted in the azocarmine stain. Collagen production appeared most marked in the corium but was almost as abundant in the muscle layers. At 6 days the wounds of deficient guinea pigs (Fig. 6, frontispiece) showed a marked exudation of fluid containing red blood cells; the exudate was seen throughout the entire extent of the wound but was most marked in the reticular layer of the corium and in the subcutis. Proliferation cells were as numerous in deficient animals as in control animals, but collagen production was definitely retarded. We were unable to detect any differences in cellular proliferation about the muscle fibers or any increase in the number of giant cells.

Striking differences in collagen production were evident when the wounds of control guinea pigs were compared with the wounds of deficient animals 8 days after operation. In many instances the wounds of animals in the control group (Fig. 7, frontispiece) were found to be almost completely replaced by mature collagen, while the wounds of deficient animals (Fig. 8, frontispiece) showed only small areas of collagen production. A marked proliferation of fibroblasts was evident in the deficient guinea pigs and only a moderate proliferation in the controls. The wounds of almost all deficient animals showed small pools of serum and red blood cells separating the wound edges. These pools were usually present in the deep layers of the corium but could be found throughout the entire wound.

At 10 days the wounds of the control group showed collagen production continued beyond that of 8 days. The wounds of deficient

animals likewise showed at 10 days a marked increase in the amount of collagen present, and, furthermore, a decrease in fibroblastic proliferation. Only by careful examination could small pools of exudate be detected in the specimens from the vitamin C deficient animals. These pools were usually found in the corium. In both the control and the deficient groups the amount of cellular débris and of foreign body reaction was markedly diminished.

In the slides of the 14 day groups there was wide variation in the amount of healing. In the majority of the control animals the wounds were almost completely healed and showed some contraction. The wound sites were difficult to detect in the corium, for the collagen was dense and the fibrocytes apparently mature. In the muscle layers a large amount of collagen was present with only a mild cellular proliferation. In deficient animals at 14 days, good wound repair was seen in some, but in many the wounds gaped at various levels. Little effusion could be seen, but marked cellular proliferation was present in most instances. The amount of collagen present, judged by azocarmine stain varied with each animal; the maximum production was usually found in the corium.

In animals deficient prior to operation but given ascorbic acid daily after operation, it was found that healing was delayed during the first 6 days. Small effusions were found in some animals, and collagen production was postponed in all. By the 8th day after operation however, the wounds appeared almost as completely healed as those of the control group.

The plasma vitamin C levels found in normal animals are lower than those reported for guinea pigs by various other workers, who, however, used titration methods. According to King the lower values are more nearly correct. This method has been in use continually in the laboratory for specimens from human patients, and gives results in the normal range (0.5 to 2.0 mgm. per 100 c.c.) when no deficiency is present. A small series of unoperated upon control animals gave results not significantly different from those for the operated upon animals. All the normal guinea

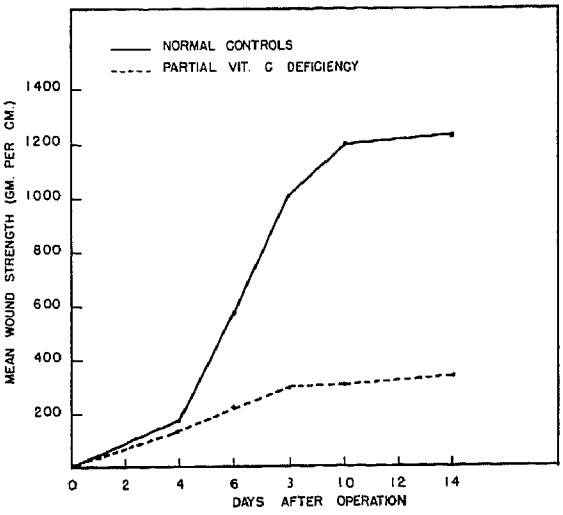

Fig. 9. Effect of partial vitamin C deficiency on wound healing in guinea pigs. The unbroken line represents the average of the tensile strength of 1 centimeter of wound in the 60 guinea pigs fed adequate amounts (5 to 10 mgm.) of ascorbic acid daily. The broken line represents the average tensile strength of 1 centimeter of wound in the 40 guinea pigs fed deficient amounts (0.2 mgm.) of ascorbic acid daily.

pigs had values above 0.05 milligram per 100 cubic centimeters, with two exceptions, and all those with deficiency had values below 0.05 with one exception.

Vitamin C intake	Plasma vitamin C
10 mgm. a day	0.22 (average of 20)
5 mgm. a day	0.13 (average of 40)
0.2 mgm. a day	0.01 (average of 40)

The average plasma protein for 60 normal guinea pigs was 4.63 per cent and for 40 partially deficient guinea pigs it was 4.55 per cent. It is seen there is no significant difference between the two groups. The values are normal for guinea pigs (8). Therefore, the lower tensile strength of the wounds in the guinea pigs fed the partially vitamin C deficient diet is not due to decreased plasma protein.

Moisture determinations on segments of the abdominal wall were made after the tensile strength had been determined in a few cases. For 7 normal animals the average was 76.1 per cent ($\pm$ 2.1 per cent) and for 6 partially vitamin C deficient animals the average was 79.1 per cent ($\pm$ 1.7 per cent). Only 1 of the deficient animals gave a result lower than the highest control.

TABLE I.—EFFECT OF PARTIAL VITAMIN C DEFICIENCY ON TENSILE STRENGTH OF WOUNDS IN GUINEA PIGS

Number of guinea pigs	Amount of vitamin administered		Tensile strength of wound in grams per centimeter				
	Ascorbic acid mgm. per day	Citrin mgm. per day	On 4th P.O. day	On 6th P.O. day	On 8th P.O. day	On 10th P.O. day	On 14th P.O. day
20	10	0	169	474	1158	1223	1120
20	5	0	189	662	1045	1198	1490
20	5	2	174	577	812	1177	1379
20	0—14th to 7th preoperative day. 0.2 mgm. thereafter	0	105	240	315	283	367
20	0—14th to 7th preoperative day, 0.2 mgm. thereafter	2	173	205	291	329	317
10	0—14th to 7th preoperative day, 0.2 mgm. to operation. 10 mgm. thereafter	0		316	909	1235	

The tensile strengths of the wounds in the guinea pigs kept on the vitamin C deficient diet and in the control animals showed a marked difference from the 6th day on. On the 4th postoperative day the differences were not significant. On the 6th day, however, the wounds of the control animals were more than twice as strong, and by the 10th day had become four times as strong as those of the deficient animals (see Fig. 9).

A study of Table I will reveal that there is no significant difference in the tensile strength between those animals kept on the daily dose of 5 milligrams of ascorbic acid, which is adequate for a guinea pig, and those kept on 10 milligrams daily, which may be considered excessive dosage.

In the last line of Table I data are presented from a group of animals which received the usual deficient amount of ascorbic acid until the time of operation. On the day after the operation they were given 10 milligrams of ascorbic acid and from then on received a daily dose of 10 milligrams. On the 6th postoperative day the tensile strengths of the wounds of these animals, while not as great as those in the control guinea pigs, were definitely above those in the deficient animals, and by the 8th postoperative day had attained the level of the controls.

The graph summarizes the results. The heavy unbroken line represents the average for the 60 guinea pigs fed adequate (5 or 10 mgm.) ascorbic acid and the broken line represents the average for 40 guinea pigs fed 0.2 milligrams of ascorbic acid daily. Each point on the unbroken line is the average for 12 animals. Each point on the broken line is the average for 8 animals. The normal wounds reached the strength of the unoperated abdominal wall by the 14th day. However, they frequently broke through elsewhere than at the wound after the 8th day. The wounds in the deficient guinea pigs always broke at the incision. The strength of the unoperated upon abdominal wall is the same in control and in deficient animals, being somewhere between 1200 and 2000 grams per centimeter.

Several normal animals were allowed to heal for 2 weeks (5 or 10 mgm. vitamin C per day). At this time the wounds were well healed. The vitamin C was then withdrawn completely, and the animals were allowed to develop scurvy of more or less severity. They were sacrificed after they had been losing weight for several days. The wounds appeared grossly to be in poor condition, with the characteristic hemorrhagic appearance, but had normal strength. Only 3 animals survived for completion of the experiment.

Sections of tissue from 2 guinea pigs sacrificed on the 20th and 22nd days following withdrawal of vitamin C showed the wounds to be well healed and comparable to those of the control animals. The tensile strengths of their wounds were 1248 and 1401 grams per centimeter of wound. The wound of the third guinea pig sacrificed on the 34th day after withdrawal of vitamin C contained a pool of serum between the muscle layers, and there was no appreciable decrease in the amount of collagen. This wound appeared comparable to those of the 8 and 10 day deficient animals, and had a tensile strength of 880 grams per centimeter of wound.

Addition of 2 milligrams per day of lemon eriodictin to the diet of either normal or partially vitamin C deficient guinea pigs had no significant effect on wound healing. (Table I). This substance is Szent-Györgyi's "vitamin P" or citrin, a flavone glycoside. It is claimed

to be useful in treating vascular forms of purpura (12), and Szent-Györgyi (3) and his associates attributed the hemorrhagic symptoms in scurvy to a deficiency of citrin. (The basal diet used in these experiments may not be entirely free from this substance). An attempt to study the capillary fragility by the method of Zacho gave somewhat erratic results, but indicated no difference between normal and partially vitamin C deficient animals or between those receiving lemon eriodictin and those not receiving it.

SUMMARY

Wound healing was found to be retarded in guinea pigs kept on a subscurvy diet. The tensile strength of wounds in these animals was about one-half normal on the 6th postoperative day and about one-quarter normal from the 8th through the 14th day.

Increasing the vitamin C dosage above the normal daily requirement did not result in greater than normal development of tensile strength in wounds.

In animals operated upon while in a subscurvy state and given a high vitamin C dosage after operation, the wounds reached the same degree of tensile strength as in normals by the 8th postoperative day.

Addition of lemon eriodictin (citrin, "vitamin P") to the diet did not improve the wound healing process in either normal or partially vitamin C deficient animals.

Histological studies showed that the low tensile strength in wounds of partially vitamin C deficient animals was due to a failure of collagen production.

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