Can the Wound Healing Process Be Improved by Vitamin Supplementation?
Experimental Study on Humans

Abstract
The improvement of the wound healing process in humans by vitamin supplements is still controversial because of the lack of a clearly demonstrated correlation with the mechanical properties of scars. Objective: The aim of this work was to study the effects of high doses of ascorbic acid (AA) and pantothenic acid (PA) on the wound healing process of human skin. Method: Two groups of patients undergoing surgery for tattoo removal by the successive resection procedure received AA (1 or 3 g/day) and PA (0.2 or 0.9 g/day). More than 80 mechanical, biological and histological parameters were investigated in both preoperated skin and the scars. Results: The breaking energy of scars was higher in group 2, and energy and treatment were directly correlated (p = 0.006). Mg and Mn significantly rose in group 2 whereas Fe decreased in a dose-dependent manner. Intragroup comparison showed patient and treatment effects for Mg, a time-treatment effect for Cu and a treatment effect for Fe. Conclusion: The degree and rapidity of variations rather than the variations of the absolute values themselves of fibroblasts, hydroxyproline, Fe, Cu and Mg are significantly related to the enhancement of the mechanical properties of scars. From this study, it may be assumed that in order to obtain 'better', more solid and resistant scars, the decrease of Fe must be quick and acute in order to avoid the harmful effects of toxic radicals; the increase of Cu, Mg and Mn must be early and high in order to have more stable and solid collagen.

Key Words
Ascorbic acid
Pantothenic acid
Wound healing
Scar
Trace elements
Fe
Cu
Zn
Mn
Mg
Introduction

In a previous study [1] the improvement of the wound healing process by pantothenic and ascorbic acids (PA and AA, respectively) was suggested possibly because of the mobilization of trace elements (Fe, Cu, Zn, Mn, and Mg) which occurred significantly in skin [2] and scars [3, 4] after supplementation. However, the lack of a direct statistical correlation between the mechanical properties of the scars themselves and the vitamin supplements, and the existence of a correlation only between trace element levels and mechanical properties did not allow definitive conclusions.

Further studies using high doses of vitamin supplements have been recommended in order to try to prove a real benefit due to vitamins; they were also necessary in order to find out significant correlations between vitamin supplements, trace element variations (rather than absolute values) and the objective improvement of the mechanical properties of scars (i.e. solidity and resistance).

The aim of this work was to study the effects of high doses of AA and PA on the wound healing process of human skin, with particular attention given to trace element variations as they are known to be involved in both the wound healing process and the mechanical properties of scars.

Patients and Methods

Twenty-seven informed patients, undergoing ambulatory surgical treatment for tattoo removal by the successive resection procedure, were asked to enter this prospective study. Inclusion and exclusion factors [1] were taken into consideration. The consent was obtained after informing the patients appropriately.

Patients were assigned to either group 1 or 2, according to their order of arrival in the ward; they were then given oral vitamin supplements. Group 1 (supplemented) comprised 17 patients given AA at 1 g/day and PA at 0.2 g/day. Group 2 (supersupplemented) comprised 10 patients given AA at 3 g/day and PA at 0.9 g/day.

The protocol included three stages within a 21-day period. First stage: on day 0, after clinical examination and blood sample collection, the patients started vitamin oral supplements. Second stage: On day 8, a first partial resection of the tattoo was performed under local anesthetic, and skin samples were removed; the wounds were closed by interrupted sutures (NYLON MONOFIL 1.5 ARCHIMED, France). Third stage: on day 21, a second surgical resection was performed, again under local anesthetic, removing both the remaining tattoo and the first scar. Blood and scar specimens were collected and vitamin supplementation was ceased.

Wound Healing Assessment

More than 80 mechanical, biological and histological criteria were analyzed [1].

Mechanical Study. Scar samples of a standardized shape were immersed in a saline solution after removal, stored at 4°C and rapidly tested in a traction device (extensometer) constructed in our laboratory [5]. The edges of the scars were fixed up to the jaws of the clamps, and an increasing force was applied on one edge of the scar until breaking occurred. The viscoelastic curve [6] was achieved by a strain gauge force transducer connected to a Philips PM 8131 recorder. Four measurements and calculations were considered [1, 2, 4, 6]:

(a) breaking strength was the force required to break the scar (g);
(b) stress was the ratio of breaking strength divided by the cross-sectional area of the scar (g/mm²);
(c) elasticity was calculated as the tangent of the angle between the x-axis and the linear part of the viscoelastic curve (no unit), and
(d) breaking energy was calculated as the surface under the disruption curve (force, g, on the y-axis, and elongation, mm on the x-axis).

Histological Study. In order to count fibroblasts of skin and scar [7], samples of a standardized shape (5 x 10 mm) were kept in Bouin’s fixative solution. These were then routinely embedded in paraffin and cut with a microtome to produce 5-μm thick sections. After Gomori staining, samples were examined with a light WILD MPS 11 microscope. The dermal fibroblasts of 36 optical fields of normal skin before the first resection procedure (day 8), and of scars on either side of the suture line after resection (day 21), were counted in each sample by using a reticular lens (× 320 magnification). Cells were considered fibroblasts according to the usual criteria; elongated shape and nucleus, and specific staining. The results were expressed as the mean number of fibroblasts per optical field.
Biochemical Study. In order to determine hydroxyproline concentration as an index of synthesis of collagen in tissues [8, 9], samples of a standardized shape (1 cm in length, 0.5 cm in width) were dissected and stored frozen at -18°C. Samples were then cut into small pieces, lyophilized and hydrolyzed in HCl. Hydroxyproline concentration of the hydrolysate was determined by a fluorimetric procedure using a BECKMAN DU 20 Fluorimeter, counting absorbance at 570 nm. Results were expressed as micrograms/milligram of dry tissue weight. This method was previously validated [6, 7] as both the scar and its surrounding area are involved in the wound healing process.

Microanalysis. In order to determine the tissular content of the main trace elements involved in the wound healing process [1-3], skin and scar samples of a standardized shape were fixed in glutaraldehyde after removal. They were washed in a cacodylate buffer, dehydrated with ethanol and acetone, and dried at the critical point using CO2 [10]. After that, samples were coated with gold in a sputter coater and examined in a Philips 501 B Scanning electron microscope at 20 kV. Microanalysis was performed using the EBS LINK systems 860. The Fe, Zn, Cu, Mn and Mg content of the tissue were expressed as counts per 100 s. This method was semiquantitative, as no absolute values and only the values of the comparisons with the tissues of the controls which have been used as reference values were available. Thereby, the only available data were the number of counts per 100 s, according to the difference between two samples of tissue. For all of these parameters, the values were calculated on days 8 and 21; then the differences between day 8 and day 21 were calculated to measure and test the influence of their real variations on the wound healing process.

Blood Tests

Blood samples were analyzed in order to determine the vitamin, nutritional, hematological and biochemical status.

Plasma ascorbic concentration (μmoles/l) was determined by using a fluorimetric procedure [11]. Thiamine (vitamin B1) and riboflavin (vitamin B2) were assessed by the measurement of the coenzyme stimulation of vitamin-dependent enzymes in the erythrocytes, and the results were expressed as the activation coefficient [12]. Vitamins A, E and β-carotene (μmoles/l) were assessed in serum by using the high-pressure liquid chromatography procedure [13]. The other tests included the determination of blood transferrin (g/l) and prealbumin (mg/l) concentrations, white and red blood cell counts, hemoglobin concentration, plasma concentration of glucose, bilirubin, and urea and hepatic enzymes.

Statistics and Ethics

Statistical analyses were performed using the Newman-Keuls test, the paired and unpaired Wilcoxon test, an analysis of variance with repeated measures, and the paired or unpaired Student t test, when necessary. Simple and multiple linear regression analyses were applied when appropriate, to study the correlations between the different parameters. Intra- and intergroup comparisons were possible by using these different tests. Furthermore, an analysis of principal components and a multifactor analysis were also performed in order to estimate the importance of the different factors presumably involved in the wound healing process.

The investigations conformed to the standards of the country where it was carried out [10].

Results

Comparison of Groups 1 and 2

There were no significant differences in age and tattoo localization. The two groups were well balanced for the usual blood parameters (hemoglobin concentration, red and white blood cell counts, plasma concentrations of proteins and hepatic enzymes). The nutritional and vitamin statuses were similar.

Effects of Supplementation of the Wound Healing Process

All of the variations observed on day 8 (supplemented skin) were confirmed on day 21 (supplemented scar).

Variations of Trace Elements in Skin and Scars

Intergroup Comparisons. In skin, after 8 days of supplementation, Mg (p = 0.0004) and Mn (p = 0.0009) increased significantly in group 2, whereas Fe decreased (p = 0.09). In scars, after 21 days of supplementation, Mg (p = 0.0016), Mn (p = 0.0009) and Cu (p =
Table 1. Intragroup (day 8–day 21) and intergroup (day 8, group 1 vs. group 2; day 21, group 1 vs. group 2) variations in skin (day 8) and scar (day 21)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day</th>
<th>FB</th>
<th>HOP</th>
<th>Mg</th>
<th>Fe</th>
<th>Cu</th>
<th>Zn</th>
<th>Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>28±7</td>
<td>39±6</td>
<td>207±97</td>
<td>568±423</td>
<td>196±84</td>
<td>55±58</td>
<td>87±38</td>
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<tr>
<td></td>
<td>21</td>
<td>90±31</td>
<td>27±6</td>
<td>270±106</td>
<td>373±188</td>
<td>145±84</td>
<td>75±66</td>
<td>87±35</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>19±7</td>
<td>36±4</td>
<td>479±236</td>
<td>280±257</td>
<td>188±107</td>
<td>84±61</td>
<td>354±118</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>80±30</td>
<td>25±8</td>
<td>448±136</td>
<td>200±92</td>
<td>264±175</td>
<td>88±61</td>
<td>341±80</td>
</tr>
</tbody>
</table>

FB = Fibroblasts, n/field; HOP = hydroxyproline, μg/mg dry tissue weight.
In counts/100 s.

Table 2. Significance in the analysis of variance for repeated measurements (group 1 vs. group 2)

<table>
<thead>
<tr>
<th>Variable effect</th>
<th>FB</th>
<th>HOP</th>
<th>Mg</th>
<th>Fe</th>
<th>Cu</th>
<th>Zn</th>
<th>Mn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>0.049</td>
<td>0.002</td>
<td>0.001</td>
<td>0.005</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>*</td>
<td>0.05</td>
<td>0.006</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td>0.22</td>
<td>0.22</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No data means no correlation. FB = Fibroblast; HOP = hydroxyproline.
*p < 0.001.

0.03) increased, whereas Fe (p = 0.02) decreased (table 1).

Intragroup Comparisons, Day 8–Day 21.
In group 1 (low doses of vitamins) Mg increased slowly but significantly (207 vs. 270, p = 0.037); in group 2 (high doses), the treatment led to a quickly and greatly increased Mg level, but the difference (day 8–day 21, 479 vs. 448) was not significant. In the analysis of variance (table 2), these phenomena explained both patient and treatment effects (p = 0.002 and 0.0008, respectively). Cu decreased in group 1 (196 vs. 145, p = 0.0084), but increased in group 2 (188 vs. 264, p = 0.22); these reversed variations explained the time-treatment effect in the analysis of the variance.

The Fe decrease was greater and significant in group 1 (567 vs. 373, p = 0.013). In group 2, the decrease was not significant (280 vs. 200, p = 0.28), but the starting level was significantly lower (= treatment effect in the analysis of variance).

Number of Fibroblasts and Hydroxyproline Variations
There were no significant differences in the increase of the number of fibroblasts and in the decrease of hydroxyproline concentrations (intergroup comparisons), which usually occur during the wound healing process (table 1). However, their variations (difference between days 8 and 21) were correlated to the breaking energy (r = -0.5, p = 0.012, and r = 0.43, p = 0.04, respectively).

Vitamin Supplements and Wound Healing

Furthermore, energy and treatment were directly correlated ($r = 0.54$, $p = 0.006$). The linear regression analysis showed that about 30% of the variation in energy was only explained by treatment. Force ($r = 0.05$, $p = 0.82$), stress ($r = 0.04$, $p = 0.85$), and elasticity ($r = -0.21$, $p = 0.33$) were not related to vitamin supplementation treatment.

### Effects of Supplementation on Vitamin Status and Plasma Data

The initial vitamin status was considered normal in the two groups (no differences between the two groups on day 0). AA plasma concentration usually decreases after surgery [14, 15]. AA supplementation led to a significant increase in plasma even in group 1 (low doses of vitamin). The AA plasma level increased from day 0 to 21 in the two groups (intragroup comparisons, $p < 0.001$). The usual negative impact of surgery was then cancelled out. Moreover, the difference between the two groups on day 0 (not significant) became significant on day 21 (intergroup comparisons, $p = 0.04$).

### Discussion

Objective criteria in order to prove the clear 'improvement' of a wound healing process are lacking; numerous protocols have failed in their endeavors because of this. Only few studies document the improvement of mechanical properties such as scar resistance [1–3]. It is very difficult to objectivize these parameters and their differences, especially on humans. Moreover the differences recorded are small, barely reaching statistical significance; in addition, the data in the literature are very often controversial and frequently present contradictory results. Basically, a treatment and time-treatment effect are expected to occur simultaneously in the analysis.
of variance to prove some beneficial effect of the vitamin supplement.

**Scar Solidity and Resistance**

The criteria scar solidity and resistance, as defined in Methods, were previously validated [1-3]. The calculation of these different parameters and the high correlation between them (table 4) confirmed the logic of the choice. Among the different criteria for scar solidity, only the breaking energy significantly rose in group 2 (135 vs. 239; p = 0.0069). This may be explained by the vitamin supplements. The other parameters, although very well correlated to each other, did not vary significantly.

**Ascorbic Acid**

AA is a cofactor of hydroxylation in the early stages of collagen synthesis and stimulates collagen-specific mRNA. AA enhances in vitro intracelular collagen synthesis [16-18], slows down its degradation, and enhances its release out of the cells [18]. However, some harmful effects (cytostatic ± cytotoxic) caused by AA have already been shown in vitro on fibroblast cultures [19], proving that no valid extrapolation can be made from in vitro experiments. The doses given in this study, although thrice higher than those routinely given by clinicians, were 10 times less than doses already given in experimental protocols without any side or adverse effects (up to 600 mg/kg/day) [20]. On the other hand, AA deficiencies clearly lead to scurvy, a serious disease accompanied by the lack of wound healing processes. However, the possibility to effectively improve the wound healing process by means of AA supplementation is still controversial.

**Pantothenic Acid**

PA effects on the wound healing process are not widely documented. PA is a coenzyme A component, actively involved in the energy production processes. In vitro, it enhances cell growth and intracellular collagen synthesis [17, 18]. In rabbits, PA supplementation improves skin, aponeurosis and colon wound healing processes by increasing fibroblast proliferation, collagen synthesis and scar resistance [2, 4]. In vitro, complementary effects of AA and PA have been highlighted [17].

**Fibroblasts and Hydroxyproline**

The increase of the number of fibroblasts and the decrease of hydroxyproline are common knowledge, no matter what wound healing process is considered. There was little hope of finding significant differences. However, their variations (day 8-day 21 and day 8/day 21) were well correlated but only as far as the breaking energy was concerned (p = 0.012 and 0.049, respectively). This seems to prove that the higher the difference (in absolute values) of the number of fibroblasts between days 8 and 21 (negative correlation, r = -0.5), the more resistant the scar is; the opposite is probably true concerning the decrease of hydroxyproline (positive correlation, r = +0.43). This (never before reported) result could be explained by the action of vitamin supplements which increase cellular growth and the release of newly formed proteins out of the cells [17, 18]. The decrease of hydroxyproline may be a sign of a high collagenase activity.

**Magnesium Metabolism**

In animals, high doses of vitamins are necessary to increase Mg levels in tissue [3, 21]. In human skin and scars, vitamin B₃ and C increase Mg counts, whereas no variation occurs without supplementation. In this study, a significant increase occurred in the supersupplemented group on day 8 (207 vs. 479; p = 0.0004), as well as on day 21 (270 vs. 448; p = 0.002). Mg is involved in collagen synthesis, and the positive role of high levels in Mg in
the wound healing process has previously been suggested [21].

Moreover, in the analysis of variance (table 2), patient and treatment effects have been demonstrated (p = 0.002 and 0.000, respectively). This strongly suggests (1) that the increase of Mg is due to vitamin supplements even before day 8, and (2) that this rise is dose-dependent.

Iron Metabolism

This study once again demonstrates that low values of Fe and high values of Cu and Zn as well as the degree of variations (difference day 8–day 21) are related to the improvement of the mechanical properties of scars. In contrast, an increased Fe content in scars seems to be related to an adverse effect on scar resistance. An Fe overload is known to have negative effects on the wound healing process [22]. The probable cause of this negative impact is the activation of the synthesis of free radicals such as hydroxyl, which alters fibroblast cell membranes and impairs their metabolic activity (i.e. collagen synthesis). The aim of vitamin supplementation then is to lead to a decrease of the Fe in tissue which can ‘improve’ the wound healing process; this is well documented in this study. Fe is the only parameter with three effects well demonstrated in the analysis of variance (patients, p = 0.001; treatment, p = 0.05; time, p = 0.006). In group 1, the decrease from day 8 to day 21 is significant (567 vs. 373, p = 0.013), but the starting level was very high; the decrease in group 2 is less important and not significant (280 vs. 200, p = 0.28), but the starting level was very low due to the high doses of vitamins previously given (intergroup comparisons on day 8: 568 vs. 280, p = 0.05). In other words, high doses of vitamins lead to a quick and important decrease of Fe in skin before surgery, of which the beneficial effects only appear later.

Copper Metabolism

In a normal wound healing process, Cu is a cofactor of lysyl oxidase, an enzyme indispensable to production of collagen of the interfibrillar bridges. It is thereby necessary for the stability and solidity of scars [16, 23]. In previous studies, Cu levels have been correlated to the mechanical resistance of human skin. Usually, an increase of Cu in blood occurs simultaneously with its decrease in tissues, especially in scars [1, 3, 21] without any vitamin supplement, but these data are still controversial. For example, Kadrabova et al. [24] have documented a decrease in Cu as well as a Cu depletion in some important organs on guinea pigs (liver, brain, testes) induced by a high-dose AA regimen; this decrease was more acute under stressful conditions [25]. In our study, the decrease of Cu in tissue was confirmed in group 1 (day 8–day 21, 196 vs. 145, p = 0.0084), but Cu cose in scars in the supersupplemented group, although not significantly (188 vs. 264, p = 0.22). However this rise in Cu between the two groups (day 8–day 21, 145 vs. 264; p = 0.03) seems to be dose-dependent. The analysis of variance (table 2) showed a patient effect (p = 0.005). It also showed a time-treatment effect (p = 0.01) which is undoubtedly the most interesting effect during a wound healing process. Besides, Cu is the only trace element for which a time-treatment effect could be documented in the analysis of variance in this study.

Zn Metabolism

Zn is a cofactor of more than 200 metalloenzymes involved in cellular growth and in protein and collagen synthesis. Zn has previously been shown moving from blood to the colon: its levels decreased in plasma whereas they increased in tissues during a colonic wound healing process [3]. This mobilization of the pool of Zn, including a drop in Zn...
serum starting very shortly after surgery, has been underlined by many authors [26, 27]. Although Zn has been shown [28] to reduce re-epithelialization and bacterial growth and to increase the inflammatory stage of the skin wound healing process, a decrease of Zn in skin induces skin lesions and impairs the skin wound healing process [26, 29]. On the other hand, it has previously been shown that protein synthesis was lower and scar formation more difficult in Zn-deficient animals [30]. Zn supplementation can improve the wound healing process [31]. It is therefore very interesting to induce an increase of Zn in scars, although it is not significant. The increase of Zn (55 vs. 84), probably induced by high doses of vitamin supplements, again seems to be dose-dependent, although not significant.

Mn Metabolism

Little has been reported on Mn variations related to the improvement of the wound healing process. The effect of wound healing on Mn variations seems to be minimal; but the fact that the increase of Mn induced by vitamin supplements is dose-dependent must be pointed out. The treatment effect (87 vs. 354, p = 0.000) is the only one to be isolated in the analysis of variance (table 2). In a previous study, a paradoxical decrease of Mn in the supplemented group was claimed to be due to doses of vitamins which were too low. This hypothesis is confirmed in the present study, in which the treatment effect is highly significant and dose-dependent.

Conclusion

In this study we were able to observe that vitamin (AA and PA) supplements acted on trace elements in human skin and scars, which is consistent with previous papers [1, 2, 21]. In spite of important individual varia-

ions, this study strongly suggests the possible positive and dose-dependent roles of AA and PA during the human skin wound healing process.

Although only one of the mechanical parameters is concerned, vitamin supplements clearly lead to an increase of the breaking energy (resistance) of the scars in a dose-dependent fashion. The supplementation also leads to an increase of Mg, Cu and Mn levels in tissues, and, concomitantly, to a decrease in the Fe level.

On the other hand, the degree and rapidity of variation, rather than the variations in the absolute values themselves of fibroblasts, hydroxyproline, Fe, Cu, and Mg, are significantly related to the enhancement of the mechanical properties of scars; these variations occur more quickly and are more acute because of vitamin supplements.

However, the link between these two events can only be suggested: it may be due to the decrease of toxic free radicals via the decrease of Fe, to the improvement of the solidity of collagen chains via the increase of Cu, and to an enhanced quality of collagen via the increase of Mg and Mn.

In other words, it appears that to have 'better', more solid and resistant scars, the decrease of Fe must be quicker and more acute in order to have fewer toxic radicals with their harmful effects. The increase of Cu, Mg and Mn must take place as early as possible and must be significant in order to obtain more stable and more solid collagen.

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References


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