Effect of vitamin C on fracture healing in elderly Osteogenic Disorder Shionogi rats

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We studied the effect of vitamin C on fracture healing in the elderly. A total of 80 elderly Osteogenic Disorder Shionogi rats were divided into four groups with different rates of vitamin C intake. A closed bilateral fracture was made in the middle third of the femur of each rat. Five weeks after fracture the femora were analysed by mechanical and histological testing. The groups with the lowest vitamin C intake demonstrated a lower mechanical resistance of the healing callus and a lower histological grade. The vitamin C levels in blood during healing correlated with the torque resistance of the callus formed ($r = 0.525$). Therefore, the supplementary vitamin C improved the mechanical resistance of the fracture callus in elderly rats. If these results are similar in humans, vitamin C supplementation should be recommended during fracture healing in the elderly.

Vitamin C is a key element in the healing of fractures. It functions in the hydroxylation of two amino acids (proline and lysine) needed to form the triple helix of collagen, which is essential for the formation of immature callus in bone healing. Vitamin C deficit can result in either delayed healing or fracture nonunion. It also has other functions in the formation of non-collagenous proteins during bone healing, as well as an important role in the development of mesenchymal, chondroblast and osteogenic phenotypes.

Vitamin C is also an important reducer. Because a fracture provokes inflammation and the generation of free radicals, reducing substances such as vitamin C may be beneficial in neutralising these harmful elements. Although some authors have reported beneficial effects, others warn that in the presence of metals such as iron, a reducing substance such as vitamin C could generate more free radicals via the Fenton reaction.

We studied 80 female Osteogenic Disorder Shionogi (ODS) rats (Clea Inc., Tokyo, Japan) aged between 12 and 13 months. These rats have a mutation in the gene coding for the production of the enzyme L-gulonogammalactone oxidase (similar to the enzyme in humans), meaning that they cannot produce their own vitamin C; they are thus suitable for the study of vitamin C deficiency.

The rats were housed at 22°C with light/dark cycles of 12 hours. They were given unrestricted access to water and a standard vitamin C-free diet. The animals were divided into five groups of two rats each, and were given dosages of 0, 0.125, 0.25, 0.5 and 1.0 mg/ml vitamin C, respectively. After seven weeks, all rats in the 0 mg/ml group had died, indicating that subclinical vitamin C deficit can seriously impair fracture healing, but the effect that subclinical hypovitaminosis C has on bone healing is unknown.

The aim of this study was to determine whether a subclinical vitamin C deficit interferes with fracture healing in an animal model, and whether healing returns to normal when supplementary vitamin C is administered.

Materials and Methods
The study followed the ethical requirements of the American Physiology Academy and was accepted and supervised by the Committee on Research and Animal Care of our centre.

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and those in the 0.125 mg/ml and 0.25 mg/ml groups showed signs of scurvy such as spontaneous bleeding and hair loss. Those in the 0.5 mg/ml and 1.0 mg/ml groups showed no symptoms of deficit, and so in the present study the dosage 0.5 mg/ml was used as the subclinical deficiency dosage.

An experimental bilateral fracture was made in the femora of all the rats. An apparatus was constructed using the principles established by Bonnarens and Einhorn. The rats were anaesthetised with an intraperitoneal injection of a solution composing 25 mg/ml of ketamine chlorhydrate (Ketolar, Parke Davis Laboratories, Barcelona, Spain), 0.1 mg/ml atropine (Atropina Braun, Braun Laboratories, Barcelona, Spain), and 0.4 mg/ml diazepam (Valium, Roche Farma Laboratories, Madrid, Spain), administered at 0.25 ml/100 mg of the animal’s weight. After anaesthesia, an antibiotic (cephazoline; Kefol, Irisfarma, Madrid, Spain) was administered at a dosage of 4 mg/100 mg of the animal’s weight into the right brachial triceps. To minimise post-operative pain after the fracture, all the rats received 0.10 mg/100 mg weight of buprenorphine 0.3 mg/ml (Buprex, Shering-Plough Laboratories, Madrid, Spain) by subcutaneous injection. Before the fracture was made an intramedullary Kirschner wire 1 mm in diameter was inserted into the right femur. For this, an anterior approach was made to the knee, lateralisising the patella and exposing the two femoral condyles. The wire was inserted through the intercondylar line without drilling. The incision was sutured with 3/0 silk and 3/0 absorbable sutures.

The fracture was made immediately afterwards and confirmed by a dental X-ray machine (dental Trophy 70 CCX, Iri Paris, France: exposure 0.10 s, 70 Kv, 80 mA) (Fig. 1). When no fracture resulted, the process was repeated, and when the fracture was not transverse the animal was removed from the experiment. After the procedure, the rats were placed in their cages, and the affected limbs were allowed to bear weight a few days later.

After the fracture was made, the two groups were again divided randomly, into four groups of 20 rats each. Two of the subgroups were maintained with the original dosage: controls with 1 mg/ml vitamin C, and vitamin C-deficient with 0.5 mg/ml. The other two subgroups received supplements: control-supplemented with 2 mg/ml (a randomly high dosage, twice the normal dose), and deficit-supplemented with 1 mg/ml (to normalise the rats with deficits).

Five weeks after the fracture, the rats were killed. The femora were removed, cleaned of soft tissue, and the intramedullary wires were taken out. None of the wires presented angulations of more than 5°. The bones were wrapped in gauze soaked in physiological salt solution and preserved at -80°C until tested.

Blood levels of vitamin C were measured during the experiment. Just before the rats were killed, blood was extracted from the jugular vein according to the method of Waynforth. The level of vitamin C was determined by high-performance liquid chromatography using the method of Koshiishi and Imanari.

The right femora were submitted to a low-speed torsion test (10°/min) using a SERVOSIS machine, model MT-10 + PCD-2k; Servosis Enterprise Pinto, Madrid, Spain), with a sensor capable of detecting pressures of less than 10 mNm. Adhesive cement (Osteopak, Biomet-Merck, Sjöbo, Sweden) was applied to the ends of the bones to fit them into the heads of the machine and avoid slippage. Each test provided the following information: XY curve (torque on the bone), maximum torque applied before breakage (maximum torque), angle deformation by maximum torque, sample rigidity calculated by the slope of the XY curve (between 10% and 50%), and energy absorbed by the sample, calculated as the area below the XY curve.

The histological test was performed with the left femora. After thawing at room temperature, the bone was fixed with buffered formol (pH 7.4) for 24 hours and decalcified with nitric acid-formalin for 24 hours. The tissue was then embedded in paraffin and sliced using a high-precision rotation Minot microtome (Leica Microsistemas SA, Barcelona, Spain), providing a series of single, thin sections (3 μm; in the sagittal plane). The sections were placed over the microscope slides, removing the paraffin and hydrating them for staining with haematoxylin and eosin. The central
section of each piece was used. The samples were digitised with a digital camera (Nikon Coolpix 4500, Barcelona, Spain) equipped with an optical microscope and a specific visor in which the areas of the different zones in each section were delimited. The different areas of the tissues that formed the fracture callus (bone, cartilage, fibre) were measured by the University of Texas Health Science Center at San Antonio image tool program. An area of every tissue was in the section. Next, a single blinded observer (TAM) examined the data and the stained slides. The fracture calluses were graded according to the amount of fibrous tissue, cartilage, woven bone and mature bone.22,23 Grade 1 indicated fibrous tissue, grade 2 predominantly fibrous tissue with some cartilage, grade 3 equal amounts of fibrous tissue and cartilage, grade 4 all cartilage, grade 5 predominantly cartilage with some woven bone, grade 6 equal amounts of cartilage and woven bone; grade 7 predominantly woven bone with some cartilage, grade 8 entirely woven bone, grade 9 woven bone and some mature bone, and grade 10 lamellar (mature) bone.

The quantitative data were expressed as mean and SD. Normality was confirmed by the Kolmogorov-Smirnov test (SPSS statistical software, Chicago, Illinois). The groups were compared with two-way analysis of variance (ANOVA). The significance level was set at p = 0.05 with the 95% confidence interval (CI) for the means being compared. In order to compare the quantities of vitamin C in the different groups of rats, an ANOVA test for multiple comparisons was performed. Pearson’s correlation coefficient was also used to determine the effect of vitamin C consumption on callus strength in the healed femur before breakage.

Results
Of the 80 initial rats, 18 died in the immediate post-operative period (< 24 hours) and four in the late post-operative period (> 24 hours). No animal was removed from the experiment because all fractures produced were transverse. In two cases the fracture process was repeated, as no fracture had been produced at the first attempt. There were 58 rats remaining by the end of the study, 16 in the vitamin C-deficient group, 11 in the deficient-supplemented group, 15 in the control group and 16 in the control-supplemented group.

The vitamin C blood levels at death varied according to the group. The vitamin C concentrations in rats before death are presented in Table I. A total of 12 samples were discarded because of errors in manipulation.

<table>
<thead>
<tr>
<th>Groups (mg/ml vitamin C in water)</th>
<th>Mean (SD)</th>
<th>95% CI</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C-deficient</td>
<td>0.54 (0.48)</td>
<td>(0.25 to 0.83)</td>
<td>13</td>
</tr>
<tr>
<td>Deficient-supplemented</td>
<td>0.63 (0.62)</td>
<td>(0.15 to 1.10)</td>
<td>9</td>
</tr>
<tr>
<td>Control</td>
<td>0.88 (0.55)</td>
<td>(0.49 to 1.28)</td>
<td>10</td>
</tr>
<tr>
<td>Control-supplemented</td>
<td>2.05 (0.59)</td>
<td>(1.72 to 2.39)</td>
<td>14</td>
</tr>
</tbody>
</table>

* 12 samples were discarded because of errors in manipulation

Figure 2 shows the positive correlation between final vitamin C values and the maximum torque values of the femora. This was significant (p < 0.05), with a correlation coefficient of \( r = 0.525 \).

The mechanical strength of the healing femora was higher in the groups with a higher intake of vitamin C. The means (SD) for torque, angle, rigidity and absorbed energy for the mechanical test for the fracture calluses are shown in Table II. The maximum torque values, which are the most critical measure of strength, are shown in Figure 3. All femora broke through the callus (White biomechanical stages I to II).24

The histological grades of the different tissues indicated slower healing, defined as a lower quantity of bone tissue and cartilage together with a higher quantity of fibrous tissue, in the vitamin-C-deficient group. The results of the histological tests are given in Table III.

All statistical comparisons between the groups are shown in Table IV.

Discussion
The results of our study indicate that bone healing was enhanced in rats with higher blood levels of vitamin C. The
The effect of vitamin C on fracture healing was studied experimentally in rats because non-invasive methods of determining the mechanical strength of the fractured callus are not yet sufficiently developed in humans. The simplest and most reproducible fracture model appears to be that of Bonnarens and Einhorn, and the best way to evaluate the healing is through mechanical testing. Of the four parameters that can be measured in the mechanical test (maximum torque, angle, rigidity and absorbed energy), the most important is maximum torque, because it indicates the maximum shear force resisted by the callus before it breaks.

Standard rats are not suitable for this study because they are able to produce their own vitamin C. Humans are unable to produce their own vitamin C, so that vitamin C blood levels solely reflect oral intake. The ODS rat is also unable to produce vitamin C, and thereby provides a good model in which to investigate changes in bone formation in a vitamin C-deficient animal. It has been shown that there is an impairment of fracture healing in ODS rats that is completely reversed when they are fed with 1 g/l of vitamin C in drinking water.

The blood levels of vitamin C were measured before the rats were killed in order to determine whether the groups were appropriate. All the measurements fell within the expected range (Table I), but the expected differences were not found when comparing the vitamin C-deficient and deficient-supplemented groups or the vitamin C-deficient and control groups, probably because of the small sample size. The sample was small because of various factors including the high cost of the animals in Europe (they were imported from Japan), which limited each group to 20 animals, the high post-operative mortality, the difficulty of extracting the serum, and the demands of the high-performance liquid chromatography test to determine the exact vitamin C levels, which caused some samples to be discarded. Despite this, the groups were sufficiently large to yield significant results and, therefore, the results of the mechanical/histological tests can be ascribed to the different quantities of vitamin C.

Of 80 rats in the study, 18 died in the immediate post-operative period, most likely because of their advanced age. The mean lifespan of these rats is unknown, but it is thought to be shorter than that of Wistar rats estimated to be 18 months. Moreover, rats from all four experimental groups died, indicating that death was not a result of vitamin C deficit.

**Table II. Mechanical test results, presented as mean (SD)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Maximum torque (Nm)</th>
<th>Angle deformation (°)</th>
<th>Rigidity (Nm/°)</th>
<th>Energy absorbed (mJ)</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C-deficient</td>
<td>0.048 (0.02)</td>
<td>17.86 (10.2)</td>
<td>0.003 (0.001)</td>
<td>0.47 (0.43)</td>
<td>16</td>
</tr>
<tr>
<td>Deficient-supplemented</td>
<td>0.077 (0.04)</td>
<td>30.11 (24.3)</td>
<td>0.004 (0.003)</td>
<td>0.99 (0.65)</td>
<td>11</td>
</tr>
<tr>
<td>Control</td>
<td>0.090 (0.04)</td>
<td>26.68 (15.9)</td>
<td>0.004 (0.004)</td>
<td>1.33 (1.02)</td>
<td>15</td>
</tr>
<tr>
<td>Control-supplemented</td>
<td>0.133 (0.07)</td>
<td>18.97 (9.25)</td>
<td>0.008 (0.005)</td>
<td>1.34 (1.16)</td>
<td>16</td>
</tr>
</tbody>
</table>

**Table III. Mean (SD) histological grade (see text) in each group**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Grade</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C-deficient</td>
<td>5.27 (1.39)</td>
<td>16</td>
</tr>
<tr>
<td>Deficient-supplemented</td>
<td>6.55 (0.69)</td>
<td>11</td>
</tr>
<tr>
<td>Control</td>
<td>6.29 (1.07)</td>
<td>15</td>
</tr>
<tr>
<td>Control-supplemented</td>
<td>6.24 (1.62)</td>
<td>16</td>
</tr>
</tbody>
</table>

Mechanical resistance of the fracture callus and the histological grade of the callus were higher in supplemented groups than in the deficient groups. Furthermore, there was a high correlation between the blood vitamin C levels at death and the mechanical resistance of the calluses formed.
It is known that clinical vitamin C deficiency (scurvy) can delay bone healing,\(^2,3\) but this study confirms that subclinical vitamin C deficiency can also delay fracture healing. More studies are needed to extend these results to humans. Given the high level of subclinical vitamin C deficit in the elderly population in developed countries,\(^13-16\) it might be helpful to prescribe dietary vitamin C supplements during bone healing.

When the control and control-supplemented groups were compared, significant differences were found in the mechanical resistance of the calluses (p = 0.044), with supplemented rats having greater resistance. However, the histological study showed no significant difference between the group supplemented above the normal dosage (control-supplemented) and those given the normal dosage (control, and deficient-supplemented).

The results of the mechanical test are in agreement with previous findings\(^9,10\) that vitamin C supplementation had positive effects on bone healing. However, those studies evaluated only the histological results. In our study we included a mechanical test, which is a rigorous way of evaluating fracture healing.\(^2,25\)

If these results are found to apply to humans, the administration of vitamin C during fracture healing may be indicated in the elderly.

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No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


