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The Effects of Vitamins E and C on Fracture Healing in Rats

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Forty-eight rats were divided into four groups of 12. For 3 days, each group received the following vitamins in once-daily doses: group 1, vitamin E only; group 2, vitamin C only; group 3, vitamins E and C; and group 4, no treatment (control). The right tibia of each rat was fractured on day 4; the rats then received the same regimen three times a week (once-daily doses) until day 21. Fracture healing was evaluated radiologically by measuring the callus indices, and histologically by a 5-point grading scale. On days 14 and 21, the callus index values in group 2 were statistically higher than those in the other groups. Histological evaluation scores in group 2 were the highest overall, and group 3 scores were higher than those in groups 1 and 4. These findings indicate that vitamin C accelerates fracture healing, vitamin E does not exert a marked effect on this process, and vitamins E and C in combination do not have a synergistic impact on fracture healing.

KEY WORDS: α-Tocopherol; Ascorbic acid; Vitamin C; Vitamin E; Rat; Fracture healing; Callus index

Introduction

In the early stage of fracture healing, in which biological interactions are observed, high concentrations of free radicals are formed because of the activation of inflammatory cells. These reactive species have unfavourable effects on the formation of granulation tissue and fracture healing.[1,2] Physiological mechanisms protect a living organism against the harmful effects of free radicals, and vitamins E and C are the most important natural anti-oxidants in non-enzymatic defence mechanisms. Several studies have proposed that the combined use of vitamins E and C increases the anti-oxidant effect of both vitamins by a synergistic mechanism.[3 – 6] In the present study, we investigated the effects of independent and combined uses of vitamins E and C on fracture healing.

Materials and methods

EXPERIMENTAL ANIMALS

In this study, the right tibiae of 48 6-month-old Sprague-Dawley rats weighing 350 – 400 g were studied. The study was registered by the Institutional Animal Ethics Committee of Uludağ University, Bursa, Turkey, and its regulations were followed in the experimental procedures.

VITAMIN DOSES AND TIBIA FRACTURE

During the experiment, room temperature was maintained at 24 ± 2 °C and access to water and food was unlimited. Rats were

divided into four groups of 12. Three groups received the following vitamins intraperitoneally (once-daily dose) for 3 days: group 1, 40 mg/kg vitamin E (α-tocopherol; Evigen, Biofarma, Istanbul, Turkey); group 2, 200 mg/kg vitamin C (L-ascorbic acid; Redoxan, Roche, Istanbul, Turkey); and group 3, a combination of 40 mg/kg vitamin E and 200 mg/kg vitamin C. From day 4, dosages were given three times a week (once-daily dose) until the end of the experiment. Group 4 was the control group, receiving no treatment.

On day 4, the right tibia of each rat was fractured under ether anaesthesia with the application of manual angulation stress, and the fractured site was left *in situ*.[7] Six rats from each group were chosen randomly and sacrificed on day 14 using high-dose penthonal given intraperitoneally. The remaining animals underwent the same procedure on day 21.

HISTOLOGICAL ANALYSIS

The right rear legs of the rats were separated at the hip, and lateral radiographs were taken, using high-resolution sheet film. The films were examined to verify that each rat had received uniform fractures at nearly the same level on the right tibia, and the fracture healing was evaluated by calculating the callus index.[8] The length of the widest part of the callus was determined in proportion to the diameter of the diaphysis measured from the intact site.

Right tibiae were removed from soft tissue without harming the callus tissue, kept for 24 h in 10% neutral buffered formalin, and fixed. Thereafter, they were decalcified in 15% aqueous formic acid for 5 days, embedded in paraffin to allow 6 µm sections to be taken through the centre of the callus. Histological sections were stained with haematoxylin and eosin.

Histological findings of the fracture healing were evaluated and scored according to the criteria defined by Allen *et al*.[7] A 5-point scale (grades 4 – 0) was used to determine the stage of fracture healing. Grade 4 indicated that bony union was complete; grade 3 indicated incomplete bony union because a small amount of cartilage was present in the callus; grade 2 described complete cartilaginous union; grade 1 described retention of fibrous elements in the cartilaginous plate (incomplete cartilaginous union); and grade 0 indicated non-union (e.g. large cavity containing blood or other fluid in the cartilaginous plate).

STATISTICAL ANALYSIS

Callus indices and histological scores were assessed using the Kruskal–Wallis test. When we established significant differences in overall assessments, the Mann–Whitney *U*-test was used to compare groups.

Results

CALLUS INDICES

The mean callus indices taken from the lateral radiographs of the right tibiae of rats sacrificed on days 14 and 21 are given in Table 1. On day 14, the mean callus indices in group 2 were significantly higher ($P < 0.05$) than those in group 3; those for group 1 were the lowest, and were similar to the control specimens.

On day 21, the mean callus indices for group 2 were significantly higher ($P < 0.05$) than the three other groups, and those for group 1 were higher than both group 3 and group 4.

HISTOLOGICAL GRADING

Histological assessments are shown in Table 2. Average grades in groups 2 and 3 were significantly higher ($P < 0.05$) than those in groups 1 and 4. There were findings of union at different levels when sections prepared from the rats sacrificed on day 21 were

TABLE 1:
Mean callus indices on days 14 and 21 as an indicator of the rate of fracture healing in rat tibiae

	Mean callus indices	
Group	**Day 14**	**Day 21**
Group 1	1.49	2.37
Group 2	2.17[a]	2.54[b]
Group 3	1.88	1.93
Group 4	1.64	1.90

[a]$P < 0.05$ versus group 3.
[b]$P < 0.05$ versus groups 1, 3 and 4.
Group 1, vitamin E only; group 2, vitamin C only; group 3, vitamins E and C; group 4, control.

TABLE 2:
Results of histological assessments on days 14 and 21 as a measure of fracture healing in rat tibiae

	Grade	
Group	**Day 14**	**Day 21**
Group 1	1.50	2.33
Group 2	2.67[a]	3.17[a]
Group 3	2.16[a]	3.00[a]
Group 4	1.67	2.00

Results are means for each group.
[a]$P < 0.05$ versus groups 1 and 4.
Group 1, vitamin E only; group 2, vitamin C only; group 3, vitamins E and C; group 4, control.

compared. There was no finding of non-union (grade 0) in any of the sections taken from rats sacrificed on day 21. In group 1, complete cartilaginous union occurred in four rats, and incomplete bony union in two; in group 2, incomplete bony union was observed in histological sections of five specimens, and complete bony union in one; in group 3, all rats had incomplete bony union; and in group 4, two had incomplete cartilaginous union, two had complete cartilaginous union and two had incomplete bony union. Histological gradings of sections taken from group 1 rats sacrificed on day 21 were higher than those in group 4 on both days 14 and 21, but were not significantly different from the other groups.

Discussion

In the first 14-day period of fracture healing in rats, osteogenic and chondrogenic cells differentiate and proliferate. After this, ossification begins, along with mineralization of the cartilage matrix in the soft callus filling the fracture gap. In our study, histological assessment and callus indices, established on days 14 and 21 following a fracture, showed that vitamin C accelerated fracture healing. For the groups in which vitamin C was used alone or in combination with vitamin E, formation and mineralization of the cartilage matrix at the fracture site were considerably quicker than those in other groups. Acceleration of

endochondral bone formation is consistent with the known biological effects of vitamin C. The transformation of undifferentiated mesenchymal cells into osteogenic and chondrogenic cells is known to be stimulated by vitamin C,[9] and this vitamin is known to accelerate the mineralization of cartilage matrix. This may be a direct effect of vitamin C, as well as being caused by an increase in the amount of collagen in the cartilage matrix or plasma 1,25-dihydroxyvitamin D_3 levels, and the number of vitamin D receptors on chondrocytes.[10 – 12]

The increase in the amount of collagen, as a direct effect of vitamin C, results in osteoblast proliferation and acceleration of bone matrix mineralization.[11,13] Here, vitamin C was observed to be effective in developing callus by accelerating new bone formation. It has been reported that vitamin C insufficiency during fracture healing might delay bone matrix mineralization.[14]

One of the best known effects of vitamin E is that it stabilizes free radicals by acting as an anti-oxidant. Bone healing may be delayed when an inducing agent is given for free-radical formation, and free radicals, effective particularly in the inflammation period, could be important in fracture healing in rats.[1] In the current study, we have not confirmed that the potential effects of vitamin E have an impact on fracture healing rate.

Synergism between vitamins E and C has been shown in *in vitro* studies, but such an interaction *in vivo* is questionable.[4,6] We have shown no difference in the rate of bone healing involving potential synergy between vitamins E and C. Experimental studies have indicated that vitamin C slows down vitamin E metabolism, and that a high intake of vitamin C raises vitamin E levels in plasma and tissues.[5,15] Burton *et al.*[16] reported that dietary vitamin C intake does not increase vitamin E levels in plasma and tissues in rats, and that there is no synergistic interaction between these vitamins. Furthermore, the nature of the interaction of these vitamins changes in different tissue types; for example, in vitamin E insufficiency, vitamin C metabolization accelerates in certain tissues.[17] We believe that further studies are needed to investigate interactions between vitamins E and C in bony tissue, under normal physiological conditions and in conditions of vitamin insufficiency.

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