SUPPLEMENTARY VITAMIN C DOES NOT ACCELERATE BONE HEALING IN A RAT TIBIA FRACTURE MODEL

VINCENZO GIORDANO[1], RODRIGO PIRES E ALBUQUERQUE[1], NEY PECEGUEIRO DO AMARAL[1], CRISTIANO CURCIO CHAME[1], FABIO DE SOUZA[1], MARA ÍBIS RODRIGUES APFEL[2]

ABSTRACT

Objective: To investigate the role of ascorbic acid supplementation on bone healing after rat tibia fracture. Methods: Thirty male Wistar rats were randomly divided into Vitamin C (Group A) and sham (Group B) groups (15 rats each). Group A received 200 mg intraperitoneally per kg per day of ascorbic acid and Group B was given saline 5 ml per kg per day intraperitoneally once a day. The animals were caged in pairs and allowed free access to tap water and a standard rodent chow ad libitum. Fractures were produced manually, they were not stabilized, and unprotected weight-bearing was allowed. At two, four, and six weeks post-fracture, the rats in both groups were anesthetized and sacrificed by cervical dislocation. Callus tissue was dissected, prepared, and analyzed histologically. Histomorphological analysis was performed at six weeks post-fracture and the extent of fracture healing was determined using a five-point scale. Results: There were no histological and histomorphological differences between drug-treated animals and the sham in the three different stages studied. By six weeks post-fracture, the five animals of each group had a complete bone union. Conclusion: Under the studied conditions, intraperitoneal Vitamin C supplementation does not accelerate the fracture healing process after experimental tibia fracture in rats. *Level of evidence: Level 2, individual study with experimental design.*

Keywords: Ascorbic acid. Fracture healing. Tibial fractures.

Citation: *Giordano V, Albuquerque RP, Amaral NP, Chame CC, Souza F, Apfel MIR. Supplementary vitamin C does not accelerate bone healing in a rat tibia fracture model. Acta Ortop Bras. 2012;20(1):*10-2. *Available from URL: http://www.scielo.br/aob.*

INTRODUCTION

Bone is a highly specialized tissue of the skeletal system.[1] As is the case in other tissues, bone consolidation involves the continuous interaction between different cell types, mediated by intrinsic and extrinsic factors.[1-3] Successful bone consolidation depends on many of these factors. The local blood supply and the patient's nutritional state are among the main factors that influence the behavior of the damaged tissue.[3,4] Recent data suggest that vitamins A, C, D and E are important activators and mediators of tissue healing.[4]

Vitamin C (or ascorbic acid) is a water-soluble vitamin and has several physiologic and pharmacological functions in the body of mammals. It is necessary for collagen formation, adequate functioning of the immune system, and as a tissue antioxidant.[4] During the proliferative phase of tissue healing, ascorbic acid is important for collagen synthesis in connective tissues due to its role of co-factor for prolinehydroxylase and lysinehydroxylase.[5] In addition, it appears to be essential for normal bone formation due to its effect on osteoblast growth and differentiation and on alkaline phosphatase expression.[5-7] Moreover, vitamin C alone or combined with vitamin E decreases platelet aggregation, thus reducing the risk of injury of the vascular endothelium and venous thrombosis.[8]

Although these studies have provided important subsidies in the definition of some functions of ascorbic acid in tissue healing, its interaction with bone regeneration has not been investigated at length.[9,10] In previous studies on fracture consolidation in guinea pigs, groups that received supplementary vitamin C demonstrated faster bone repair than control groups. At the molecular level, it appears that high concentrations of Vitamin C increase type X collagen expression, thus accelerating the mineralization process of the fracture consolidation.[10,11]

To elucidate the role of ascorbic acid supplementation in bone consolidation, its effect on osteogenesis was investigated in an experimental model of rat tibial fracture.

All the authors declare that there is no potential conflict of interest referring to this article.

1 - Serviço de Ortopedia e Traumatologia Prof. Nova Monteiro, Hospital Municipal Miguel Couto – Rio de Janeiro, RJ, Brazil.
2 - Department of Histology and Embryology, Instituto de Biologia Roberto Alcântara Gomes (IBRAG), Biomedical Center, UniversidadeEstadual do Rio de Janeiro Rio de Janeiro, RJ, Brazil.

Study conducted at Serviço de Ortopedia e Traumatologia Prof. Nova Monteiro, Hospital Municipal Miguel Couto, and at the Department of Histology and Embryology, Instituto de Biologia Roberto Alcântara Gomes (IBRAG), Biomedical Center, Universidade Estadual do Rio de Janeiro, Rio de Janeiro, RJ, Brazil.
Mailing Address: Rua Aristides Espínola 11/301, Leblon, Rio de Janeiro, RJ.CEP 22440-050, Brazil. E-mail: sot.hmmc@terra.com.br - v_giordano@mac.com

Article received on 11/19/10 and approved on 1/12/11.

METHODS

Thirty male mice Wistar rats (Rattus norvegicus albinus), with average body weight of 100g, were used in the experiment. The animals were randomly divided into "vitamin C" (Group A) and "sham" (Group B) groups, with 15 rats in each group. The animals were separated into pairs and put in cages, with water and standard feed ad libitum (Nuvilab CR-1, Nuvital Nutrientes, Brazil). The animal feed used did not contain vitamin C. Group A received daily intraperitoneal injections of 200mg/kg of ascorbic acid (Laboratório Teuto Brasileiro, Brazil). The dose of ascorbic acid was calculated with a basis on a study by Sarisözen et al.[9] Group B received daily intraperitoneal injections of 5ml/kg of sterile saline solution. The treatments were started on the day of the fracture and were interrupted one day before euthanasia.

The rats were anesthetized with ether and the fracture was produced manually in the middle third of the tibia using a three-point flexion technique.[2] The fractures were not stabilized, and weight bearing without immobilization was allowed as soon as the animals had recovered from the anesthesia. At two, four and six weeks after the fracture, the rats in both groups were anesthetized and euthanized by cervical displacement. This method is recommended by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.[12] The fractured extremities were dislocated at knee level to facilitate the callus preparation. The bone callus was dissected and fixed in 10% paraformaldehyde for five days.

The histological analysis was performed by light microscopy. After fixation, the fragments were decalcified in 5% nitric acid for five days, dehydrated in ethanol and embedded in paraffin. Five-micrometer cross sections were prepared, placed on slides and stained with hematoxylin and eosin. This method was described by Bancroft and Cook and was used in other experiments.[2,13-16]

The histomorphological analysis was carried out at the end of the experiment (six weeks after fracture). The callus of five rats from each group was studied randomly without knowing the treatment regime to which it had been submitted and the fracture consolidation stage was determined using a five-point scale proposed by Allen et al.[17] (Table 1)

The Mann-Whitney test was used for comparison between groups with significance level $\alpha = 0.05$.

Table 1. *Fracture consolidation score – Grading system of Allen et al.*[17]

Histomorphological evaluation	Degree
Complete bone union	4
Incomplete bone union	3
Complete cartilage union	2
Incomplete cartilage union	1
Pseudoarthrosis	0

Source: Allen HL, Wase A, Bear WT. Acta OrthopScand, 1980.

RESULTS

The consolidation of fractures in both groups occurred in a manner similar to that described by Udupa and Prasad.[18] We did not observe histological and histomorphological differences between the animals treated with the drug and those from the sham group throughout the three different stages used in the experiment ($p > 0.05$).

Two weeks after the fracture, the histological analysis showed mixed tissue callus characterized by small quantities of cartilaginous tissue and acid proteoglycans with newly formed endochondral and intramembranous bone in the connective tissue existing between the fractured bone extremities. Four weeks after the fracture, there was an extensive zone of primary bone neoformed by osteochondral and intramembranous ossification with few areas of hyaline cartilage (chondrogenic foci) detectable on the fringe of the callus. The periosteum was fully proliferative. Six weeks after the fracture, there was complete evidence of fracture consolidation. A large quantity of immature bone was noticed, with dense and irregular trabeculae. In the peripheral region, there were clear signs of remodeling of the external callus and the periosteum was relatively thin. According to the grading system of Allen et al.[17], the five animals of each group had complete bone union in six weeks (Degree 4). The bone consolidation sequence in Group A is illustrated in Figure 1.

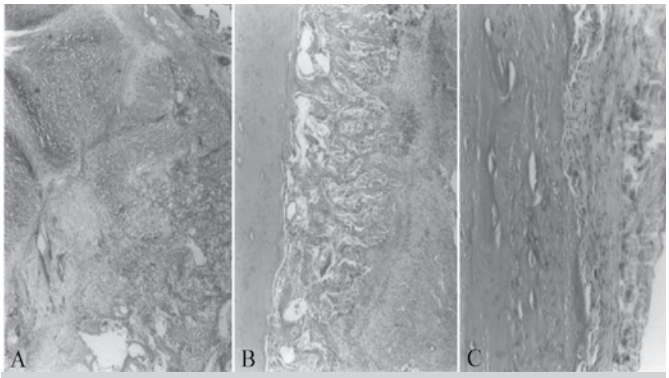

Figure 1. *Photomicrography of tibial fracture of rats in Group A (Vitamin C) at two (A), four (B) and six (C) weeks. Note the progression of the tissue callus over the period of the collagen (A), osteogenic (B) and remodeling (C) phases, as described by Udupa and Prasad.18 Complete bone consolidation was detectable at six weeks after the fracture.*

DISCUSSION

Vitamin C is important in the metabolism of several tissues of the body, especially in the formation of collagen fibers.[19] It was demonstrated that ascorbic acid promotes the synthesis of mature and normal collagen through the perfect maintenance of the activity of the enzymes lysyl hydroxylase and prolyl hydroxylase.[19,20] In an intracellular environment these enzymes catalyze the hydroxylation of some lysine and proline residues in the collagen polypeptides, enabling the formation and stabilization of the collagen triple helix.[19,21] Franceschi et al.[22] demonstrated a specific sodium-dependent carrier for ascorbic acid in the plasmatic membrane of the osteoblast, thus indicating a function of the nutrient as a Type I collagen synthesis

stimulating agent for this cell population. Moreover, ascorbic acid stimulates the action of alkaline phosphatase and induces the osteoblastic differentiation of ST2 stromal cells in vitro.[23]

Several clinical experiments prove the role of ascorbic acid in bone tissue formation. In younger populations, lack of vitamin C changes the formation of the bone matrix and cartilage resorption, leading to bone fragility and growth plate fractures.[24] It appears that activities of the chondrocytes and osteoblasts are hindered by the deficiency of ascorbic acid due to the build-up of non-helical, nonhydroxylated procollagen in the wrinkled endoplasmic reticle.[5,25] In fact, Ganta et al.[5] observed mineralization of irregular format with a randomly distributed layer of poorly formed osteoblasts on parietal bones of rat fetus treated with low doses of ascorbic acid. On the other hand, Braddock et al.[11] showed that treatment with ascorbic acid consists of an effective measure to improve skeletal ossification in diabetic rat fetuses, possibly via reduction of oxygen free radicals. Although the mechanism whereby reactive oxygen species affect the bone physiology remains unclear, it was demonstrated that oxygen free radicals are detrimental to fracture consolidation in rats.[26] Furthermore, it seems that ascorbic acid plays a crucial role in homeostasis between osteoblasts and osteoclasts in terms of differentiation and activation, directly influencing the initial stages of bone repair.[27] However, although Sarisözen et al.[9] and Yilmaz et al.[10], using experimental models of fracture in rats, have concluded that vitamin C accelerates fracture consolidation,as far as we know, no clinical or radiological benefit in the bone metabolism was consistently described with a higher dose of vitamin C. In the current experience, supplementary vitamin C in the diet did not alter bone repair in rat tibial fractures.

Unlike humans, other primates and guinea pigs, whose liver does not contain an enzymatic system that converts glucuronic acid derived from glucose into ascorbic acid, rats can obtain sufficient vitamin C from standard rodent feed.[28-30] Rats exposed to a normal diet produce between 2.8mg and 13.9mg of vitamin C per day. In our model, all the animals received standard rodent feed ad libitum and were able to synthesize ascorbic acid normally from this diet. Bourne and MacKinnon[31] did not verify an improvement in the bone consolidation of rats with an adequate diet when vitamin C was injected subcutaneously. Pointillart et al.[32] demonstrated that ascorbic acid supplementation did not positively influence bone mineral content and mineral absorption in growing pigs. Although Sarisözen et al.[9] and Yilmaz et al.[10] have not specified which type of feed was used in their investigations, it appears unlikely that rats receiving the same type of feed could benefit from vitamin C supplementation. Based on the vast literature about the benefits of vitamin C in the bone metabolism, we believe that ascorbic acid supplementation might be beneficial in the repair of fractures in species that do not synthesize this nutrient. The use of rats does not appear appropriate for this investigation. We should conduct studies in more depth using animals that do not have the ability to produce vitamin C.

In short, the data presented here demonstrate that, in experimental bilateral tibial diaphyseal fractures in rats, the intraperitoneal supplementation of vitamin C does not change the fracture consolidation process.

REFERENCES

1. Giordano V, Giordano M, Knackfuss IG. Fatores de crescimento e diferenciação ósseos. Efeitos sobre o processo de consolidação óssea: presente e futuro. Rev Bras Med. 2000;57:1018-29.
2. Giordano V, Giordano M, Knackfuss IG, Apfel MI, Gomes, RD. Effect of tenoxicam on fracture healing in rat tibiae. Injury. 2003;34:85-94.
3. Broughton G 2nd, Janis JE, Attinger CE. Wound healing: an overview. PlastReconstr Surg. 2006;117(7 Suppl):1e-S-32e-S.
4. MacKay D, Miller AL. Nutritional support for wound healing. Altern Med Rev. 2003;8:359-77.
5. Ganta DR, McCarthy MB, Gronowicz GA. Ascorbic acid alters collagen integrins in bone culture. Endocrinology. 1997;138:3606-12.
6. Franceschi RT, Iyer BS, Cui Y. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. J Bone Miner Res. 1994;9:843-54.
7. Harada S, Matsumoto T, Ogata E. Role of ascorbic acid in the regulation of proliferation in osteoblast-like MC3T3-E1 cells. J Bone Miner Res. 1991;6:903-8.
8. Mehta J, Li D, Mehta JL. Vitamins C and E prolong time to arterial thrombosis in rats. J Nutr. 1999;129:109-12.
9. Sarisözen B, Durak K, Dinçer G, BilgenOF. The effects of vitamins E and C on fracture healing in rats. J Int Med Res. 2002;30:309-13.
10. Yilmaz C, Erdemli E, Selek H, Kinik H, Arikan M, Erdemli B. The contribution of vitamin C to healing of experimental fractures. Arch Orthop Trauma Surg. 2001;121:426-8.
11. Braddock R, Simán CM, Hamilton K, Garland HO, Sibley CP. Gamma-linoleic acid and ascorbate improves skeletal ossification in offspring of diabetic rats. Pediatr Res. 2002;51:647-52.
12. Close B, Banister K, Baumans V, Bernoth EM, Bromage N, Bunyan J, et al. Recommendations for euthanasia of experimental animals: Part 1. DGXI of the European Commission. Lab Anim. 1996;30:293-316.
13. Brancoft JD, Cook HC. Manual of histological techniques and their diagnostic application.New York:Churchill/Livingstone; 1994.
14. Giordano V, Fidelis J, Giordano M, Amaral NP, Sayão A, Frediani R, et al. Não existe diferença entre tenoxicam e meloxicam na consolidação e remodelação de fraturas. Estudo histológico em tíbia de ratos. RevBras Ortop. 2003;38:201-12.
15. Giordano V, KnackfussIG, Gomes RC, Giordano M, Mendonça RG, Coutynho F. Influência do laser de baixa energia no processo de consolidação de fratura de tíbia: estudo experimental em ratos. RevBras Ortop. 2001;36:174-8.
16. Padula EOC, Andrade ML, Giordano V, Ramalho MV. Aspectos morfológicos do processo de consoolidação de fraturas em ratos diabéticos. Rev Bras Ortop. 2003;38:127-36.
17. Allen HL, Wase A, Bear WT. Indomethacin and aspirin: effect of nonsteroidal anti-inflammatory agents on the rate of fracture repair in the rat. ActaOrthop Scand. 1980;51:595-600.
18. Udupa KN, Prasad GC. Chemical and histochemical studies on the organic constituents in fracture repair in rats. J Bone Joint Surg Br. 1963;45:770-9.
19. Manela-Azulay M, Mandarim-de-Lacerda CA, Perez MA,Filgueira AL, Cuzzi T.Vitamin C. An Bras Dermatol. 2003;78:265-72.
20. Phillips CL, Combs SB, Pinnell SR. Effects of ascorbic acid on proliferation and collagen synthesis in relation to the donor age of human dermal fibroblasts.J Invest Dermatol. 1994;103:228-32.
21. Termine JD, Robey PG. Bone matrix proteins and the mineralization process. In: Favus MJ editor. Primer on the metabolic bone diseases and disorders of mineral metabolism. Philadelphia:Lippincott-Raven; 1996. p.24-8.
22. Franceschi RT, Wilson JX, Dixon SJ. Requirement for Na(+)-dependent ascorbic acid transport in osteoblast function. Am J Physiol. 1995;268(6Pt1):C1430-9.
23. Otsuka E, Yamaguchi A, Hirose S, Hagiwara H. Characterization of osteoblastic differentiation of stromal cell line ST2 that is induced by ascorbic acid. Am J Physiol. 1999;277(1Pt1):C132-8.
24. Weinstein M, Babyn P, Zlotkin S. An orange a day keeps the doctor away:scurvy in the year 2000. Pediatrics. 2001;108:E55.
25. Pacifici M, Iozzo RV. Remodeling of the rough endoplasmic reticulum during stimulation of procollagen secretion by ascorbic acid in cultured chondrocytes. A biochemical and morphological study. J Biol Chem. 1988;263:2483-92.
26. Göktürk E, Turgut A, Bayçu C, Günal I, Seber S, Gülbas Z. Oxygen-free radicals impair fracture healing in rats. ActaOrthop Scand. 1995;66:473-5.
27. Otsuka E, Kato Y, Hirose S, Hagiwara H. Role of ascorbic acid in the osteoclast formation: induction of osteoclast differentiation factor with formation of the extracellular collagen matrix. Endocrinology. 2000;141:3006-11.
28. Holford P. Vitamin C: how much is enough. Disponível em: http://www.vitamincfoundation.org/ mega_1_1.html#HOLFORD. Acesso em: 07 Maio 2011.
29. Smith D, Shang F, Nowell TR, Asmundsson G, Perrone G, Dallal G, et al. Decreasing ascorbate intake does not affect the levels of glutathione, tocopherol or retinol in the ascorbate-requiring osteogenic disorder shionogi rats. J Nutr. 1999;129:1229-32.
30. Górska P. Principles in laboratory animal research for experimental purposes. MedSciMonit. 2000;6:171-80.
31. Bourne G, MacKinnon M. The effect of ascorbic acid (Vitamin C), calcium ascorbate, and calcium gluconate on the regeneration of bone in rats. Q J ExpPhysiolCogn Med Sci. 1942;31:319-31.
32. Pointillart A, Denis I, Colin C, Lacroix H. Vitamin C supplementation does not modify bone mineral content or mineral absorption in growing pigs. J Nutr.1997;127:1514-8.