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Basic Research

Postoperative Administration of Alpha-tocopherol Enhances Osseointegration of Stainless Steel Implants: An In Vivo Rat Model

Matthaios Savvidis MD, Kyriakos Papavasiliou MD, PhD, Ioannis Taitzoglou PhD,
Aggeliki Giannakopoulou MD, PhD, Dimitrios Kitridis MD, MSc, Nikiforos Galanis MD, PhD,
Ioannis Vrabas PhD, Eleftherios Tsiridis MD, PhD

Abstract

Background Alpha-tocopherol, a well-known antioxidative agent, may have a positive effect on bone formation during the remodeling phase of secondary fracture healing. Fracture healing and osseointegration of implants share common biological pathways; hence, alpha-tocopherol may enhance implant osseointegration.

M. Savvidis, 2nd Orthopaedic Department, 424 Army General Training Hospital, Thessaloniki, Greece

K. Papavasiliou, N. Galanis, E. Tsiridis, 3rd Orthopaedic Department, Aristotle University of Thessaloniki Medical School, Papageorgiou General Hospital, Thessaloniki, Greece

I. Taitzoglou, Laboratory of Physiology, University Campus, Aristotle University of Thessaloniki School of Veterinary Medicine, Thessaloniki, Greece

A. Giannakopoulou, Department of Hematology, G. Papanicolaou General Hospital, Thessaloniki, Greece, and Laboratory of Anatomy, Histology and Embryology, Aristotle, University of Thessaloniki School of Veterinary Medicine, Thessaloniki, Greece

D. Kitridis, 1st Orthopaedic Department, George Papanikolaou General Hospital of Thessaloniki, Thessaloniki, Greece

I Vrabas, School of Physical Education and Sport Science at Serres, Aristotle University of Thessaloniki, Serres, Greece

M. Savvidis (✉), 2nd Orthopaedic Department, 424 Army General Training Hospital, Thessaloniki Ring Road West, 564 29 Nea Efkarpia, Thessaloniki, Greece, Email: makisorto@hotmail.com

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Questions/purposes This experimental study in rats assessed the ability of alpha-tocopherol to enhance osseointegration of orthopaedic implants as determined by (1) pull-out strength and removal torque and (2) a histomorphological assessment of bone formation. In addition, we asked, (3) is there a correlation between the administration of alpha-tocopherol and a reduction in postoperative oxidative stress (as determined by malondialdehyde, protein carbonyls, reduced and oxidized glutathione and their ratio, catalase activity and total antioxidant capacity) that develops after implantation of an orthopaedic implant?

Methods This blinded study was performed in study and control groups, each consisting of 15 young adult male Wistar rats. On Day 0, a custom-designed stainless-steel screw was implanted in the proximal metaphysis of both tibias of all rats. On Day 1, animals were randomized to receive either alpha-tocopherol (40 mg/kg once per day intraperitoneally) or saline (controls). Animals were treated according to identical perioperative and postoperative protocols and were euthanized on Day 29. All animals completed the study and all tibias were suitable for evaluation. Implant pullout strength was assessed in the right tibias, and removal torque and histomorphometric evaluations (that is, volume of newly formed bone surrounding the implant in mm^3, percentage of newly formed bone, percentage of bone marrow surrounding the implant per optical field, thickness of newly formed bone in μm, percentage of mineralized bone in newly formed bone, volume of mature newly formed bone surrounding the implant in mm^3 and percentage of mineralized newly formed bone per tissue area) were performed in the left tibias. The plasma levels of alpha-tocopherol, malondialdehyde, protein carbonyls, glutathione, glutathione disulfide, catalase, and the total antioxidant capacity were evaluated, and the ratio of glutathione to oxidized glutathione was calculated.

Results All parameters were different between the alpha-tocopherol-treated and control rats, favoring those in the alpha-tocopherol group. The pullout strength for the alpha-tocopherol group (mean ± SD) was 124.9 ± 20.7 newtons (N) versus 88.1 ± 12.7 N in the control group (mean difference -36.7 [95% CI -49.6 to -23.9]; p < 0.001). The torque median value was 7 (range 5.4 to 8.3) versus 5.2 (range 3.6 to 6) N/cm (p < 0.001). The newly formed bone volume was 29.8 ± 5.7 X 10^{-3} versus 25.2 ± 7.8 X 10^{-3} mm^3 (mean difference -4.6 [95% CI -8.3 to -0.8]; p = 0.018), the percentage of mineralized bone in newly formed bone was 74.6% ± 8.7% versus 62.1% ± 9.8% (mean difference -12.5 [95% CI -20.2 to -4.8]; p = 0.003), the percentage of mineralized newly formed bone per tissue area was 40.3 ± 8.6% versus 34.8 ± 9% (mean difference -5.5 [95% CI -10.4 to -0.6]; p = 0.028), the glutathione level was 2 ± 0.4 versus 1.3 ± 0.3 $\mu mol/g$ of hemoglobin (mean difference -0.6 [95% CI -0.9 to -0.4]; p < 0.001), the median glutathione/oxidized glutathione ratio was 438.8 (range 298 to 553) versus 340.1 (range 212 to 454; p = 0.002), the catalase activity was 155.6 ± 44.6 versus 87.3 ± 25.2 U/mg Hb (mean difference -68.3 [95% CI -95.4 to -41.2]; p < 0.001), the malondialdehyde level was 0.07 ± 0.02 versus 0.14 ± 0.03 $\mu mol/g$ protein (mean difference 0.07 [95% CI 0.05 to 0.09]; p < 0.001), the protein carbonyl level was 0.16 ± 0.04 versus 0.27 ± 0.08 nmol/mg of protein (mean difference -0.1 [95% CI 0.05 to 0.15]; p = 0.002), the alpha-tocopherol level was 3.9 ± 4.1 versus 0.9 ± 0.2 mg/dL (mean difference -3 [95% CI -5.2 to -0.7]; p = 0.011), and the total antioxidant capacity was 15.9 ± 3.2 versus 13.7 ± 1.7 nmol 2,2-diphenyl-1-picrylhydrazyl radical/g of protein (mean difference -2.1 [95% CI -4.1 to -0.18]; p = 0.008).

Conclusions These results using an in vivo rat model support that postoperatively administered alpha-tocopherol can enhance the osseointegration of an orthopaedic implant, although a cause and effect relationship between the administration of alpha-tocopherol and a reduction in postoperative stress cannot be securely established.

Clinical Relevance These findings suggest that postoperative administration of alpha-tocopherol is a promising approach to enhance osseointegration of orthopaedic implants in patients. Further studies with different animal models and/or different implants and those evaluating the alpha-tocopherol dose response are needed before performing clinical trials that will examine whether these promising, preliminary results can be extrapolated to the clinical setting as well.

Introduction

Oxygen free radicals are considered the major common and final pathway of tissue injury in different organ systems [43]. Oxygen free radicals contribute to the development of oxidative stress, which is a chain of oxidative events that leads to increased production of reactive oxygen species that cause tissue injury [51]. After a fracture, oxidative stress injury occurs and may be caused by an ischemia-reperfusion mechanism [26, 38, 45]. Activated polymorphonuclear neutrophils produce oxygen free radicals during the inflammatory phase of fracture healing [4, 49]. This process is further enhanced by impairment of the blood supply to the bone ends [4, 41]. These free radicals also inhibit fracture healing [4, 20] by initiating a chain reaction that will cause lipid peroxidation, which in turn leads to cell membrane damage and eventually cell lysis [4, 49]. Implantation of an orthopaedic device leads to the same ischemia-reperfusion pattern seen after a fracture, at least regarding the initial host response [33, 46, 55, 56].

As a result, oxidative stress is likely to occur after implantation of an orthopaedic device.

Vitamin E is a lipid-soluble molecule with well-known antioxidant properties [4, 7], which protects the cellular membranes by reacting with lipid radicals produced during lipid peroxidation chain reactions [4, 68]. Among vitamin E isomers, alpha-tocopherol has the highest bioavailability [1, 50] because it is absorbed by the body [5, 50, 59]. Furthermore, alpha-tocopherol may exert a substantial (potentially positive) effect on bone formation during the normal bone remodeling phase of secondary fracture healing, thus enhancing fracture healing [17, 34]. Because fracture healing and implant osseointegration share common biological pathways (such as osteoinduction and osteoconduction) [2], alpha-tocopherol may enhance implant osseointegration as well.

This experimental study in rats aimed to assess the ability of alpha-tocopherol to enhance osseointegration of orthopaedic implants as determined by (1) pull-out strength and removal torque and (2) a histomorphological assessment of bone formation. In addition, we asked, (3) is there a correlation between the administration of alpha-tocopherol and reduction in postoperative oxidative stress (as determined by malondialdehyde, protein carbonyls, reduced and oxidized glutathione and their ratio, catalase activity and total antioxidant capacity) that develops after implantation of an orthopaedic implant.

Materials and Methods

This blinded, in vivo animal study was approved by the Department of Animal Health and Welfare, Veterinarian Drugs, and Applications of our institution (Ref. 400864/4401; December 30, 2014) and it was performed in alpha-tocopherol-treated and control groups, each consisting of 15 young adult 12-week-old male Wistar rats. This study was performed in male rats only, since gender is a factor that affects the response to oxidative stress and female rats often suffer less oxidative damage than males [8]. We used the same pharmaceutical perioperative protocol for all animals, which had unrestricted access to commercial food and water, lived in an environment with a controlled temperature (22°C) and humidity (50%), and were exposed to the same period of light daily (12 hours).

On Day 0, a custom-designed stainless steel 316 L screw (Sanmac®, Sandviken, Sweden) was implanted in the proximal metaphysis of the tibias, with the rats under general anesthesia (Fig. 1A-B). The screw was manufactured to adapt to the length of the transverse axis of the rat tibia (which is < 7 mm) and to penetrate the medial but not the lateral cortex. Anesthesia was induced with intraperitoneal ketamine, 70 mg/kg of body weight
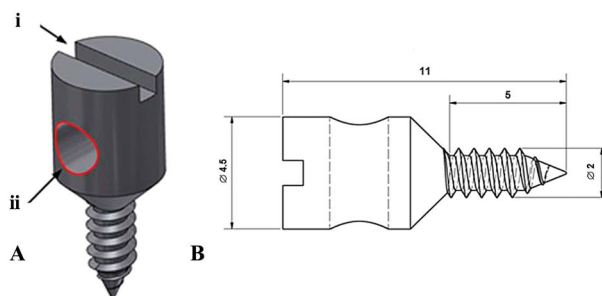

Fig. 1 A-B This figure shows the custom-designed 316 L (Sanmac®, Sandviken, Sweden, ASTM: MT 316L) stainless steel screw that was used to evaluate pull-out strength and torque. (**A**) This illustration of the screw shows an indentation (i) for the screwdriver and a hole (ii) that was used to fix the screw to the test rigs. (**B**) This image is a graphic diagram of the screw (Scale bar = 2 mm, all dimensions are in mm).

(Ketaset®, Pfizer/FDAH, Fort Dodge, IA, USA) and xylazine, 10 mg/kg of body weight (Sedaxylan®, Bimeda, Dublin, Ireland) [10]. Preoperatively, as antibiotic prophylaxis, the animals also received one intraperitoneal dose of 10 mg/kg of body weight of enrofloxacin 0.5% (Baytril®, Bayer Animal Health GmbH, Leverkusen, Germany). A self-drilling and self-tapping screw was implanted 0.5 cm distally to the superior articular surface of the tibia and perpendicular to the long axis of the tibia and was directed from medially to laterally (Fig. 2A-B). The entry point of the screw was made with a 21-gauge needle. Analgesia was administered via four doses of 80 μg/kg body weight of fentanyl (Sublimaze®, Janssen-Cilag, Macquarie Park, Australia). The first dose was given immediately before sedation, the second after the procedure was completed, and the final two every 15 minutes thereafter [10]. The wound was closed with a nonabsorbable 3-0 suture (Prolene®, Ethicon/Johnson & Johnson, Cornelia, GA, USA). The sutures were removed on Day 10.

On Day 1, animals were randomly assigned [29] to receive either 40 mg/kg body weight of alpha-tocopherol (Nepalm Vitamine E, 100 mg/²ml, Cenexi, France) once per day intraperitoneally (alpha-tocopherol group) or the same volume of saline (control group). The alpha-tocopherol and saline injections were prepared and administered daily. All animals were labeled in a blinded manner to ensure that we were not aware of which animal belonged to which group. Each animal was weighed at the end of each week of the experiment, and the dosage of alpha-tocopherol or saline was adjusted accordingly. On Day 29, all animals were euthanized by exsanguination (cardiac puncture) under general anesthesia, using the same procedure as was used preoperatively [3, 10]. After the study was completed, the results of each animal were unblinded and statistically evaluated by a third-party, nonauthor statistician (AT).
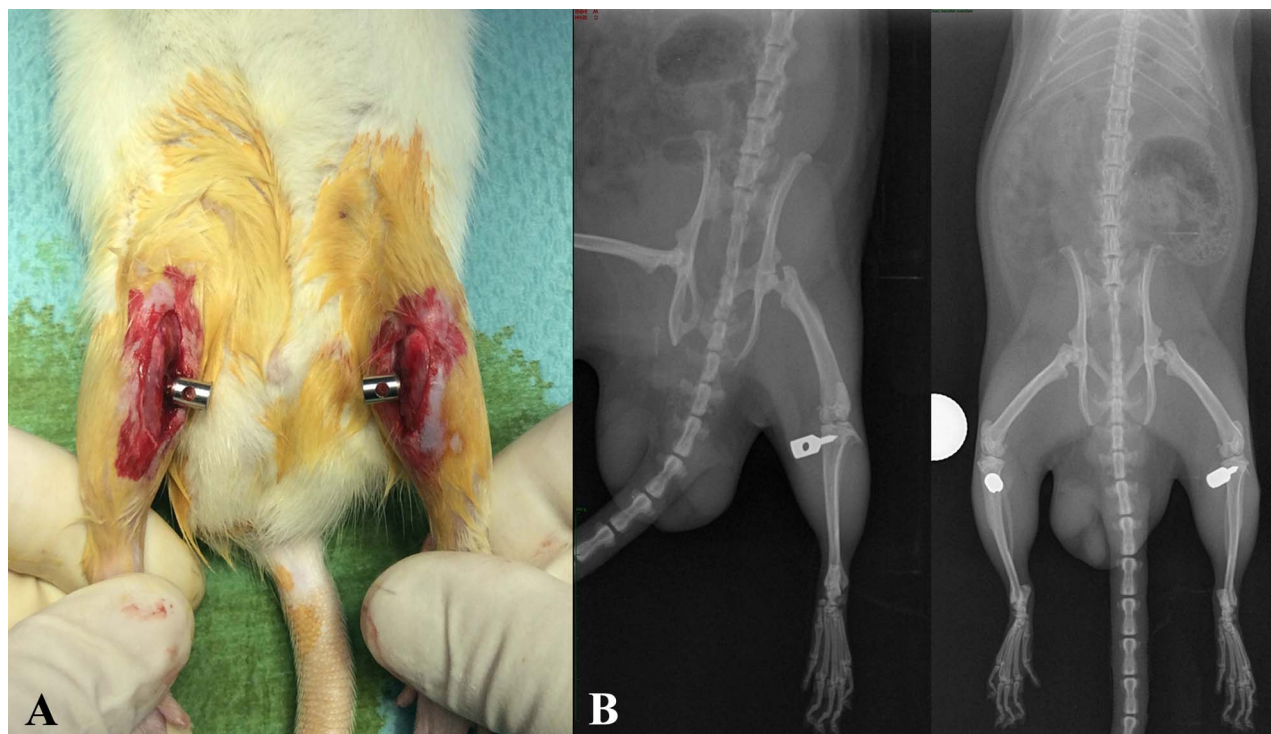
Fig. 2 A-B (**A**) This intraoperative photograph shows the position of the screws in the proximal tibias of the rats. (**B**) These standard latero-lateral and AP radiographs were taken immediately postoperatively and depict the proper position of the stainless steel screw at the proximal tibia.

Osseointegration Evaluation

After the rats were euthanized, both tibias were harvested and stripped clear from the surrounding soft tissues. They were placed in a gauze dressing that was soaked in ambient-temperature saline. The implants' pullout strength and torque were assessed within the first 2 hours after the animals were euthanized, using a custom-designed and manufactured device named "Procroustis" (Laboratory for Machine Tools and Manufacturing Engineering, Department of Mechanical Engineering, Aristotle University of Thessaloniki, Thessaloniki, Greece). This device (Fig. 3A) automatically records the applied force during pulling out (Fig. 3B) or rotating (Fig. 3C) of the screw that is inserted into the bone. The force and the displacement are recorded and displayed as a diagram at the monitor of a computer that is part of the whole device. The force is measured by a Kistler dynamometer (Kistler Group, Winterthur, Switzerland) that is placed under the movement placard of the piston and above the penetrator. The analog signal of the measurement device is turned into a digital signal by an AT converter. The digital control, the data processing, the recording and the results presentation were achieved by a program based on Labview software (National Instruments, Austin, TX, USA). The device can run torsion tests after proper modification (Fig. 3C).

Pullout strength was measured in right tibias and maximum removal torque was measured in left tibias.

Histomorphometric Analysis

After assessing torque in each left tibia, we fixed the bone (with the screw still implanted, since during the torque test, only a slight rotation of the screw was applied until the torque measurement was completed) in a buffered formalin solution (10%) for 1 month in preparation for histomorphometric analysis. Then, tibias were washed in tap water and demineralized in hydrochloric acid (< 7%) for 5 to 8 days. Subsequently, the implants were carefully removed from the bone and tibias were washed in running water for 6 hours, dehydrated in a series of graded alcohol solutions and processed for embedding in paraffin. Semi-serial sections with a thickness of 7 μm were cut along the major axis of the tibia (longitudinal sections) and a plane parallel to the long axis of the screw cavity, using thin-sectioning microtomy (Microm HM315, Rankin Biomedical Corp, Holly, MI, USA). One in 20 consecutive sections (18 to 22 sections) contained in the central part of the implant site were collected and used to estimate each parameter. Sections were placed on superfrost-plus slides (Thermo Scientific, Braunschweig, Germany) for
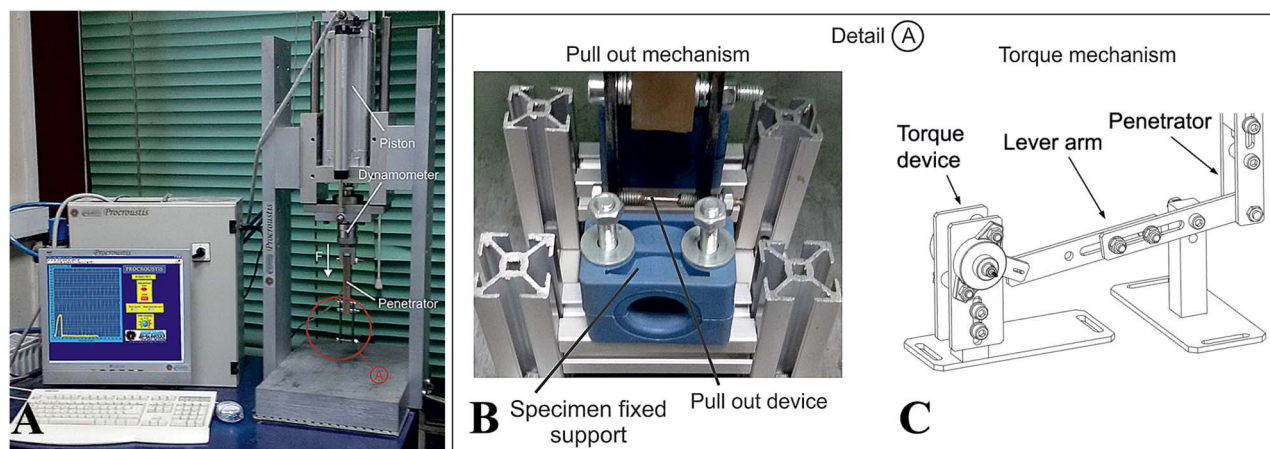
Fig. 3 A-C (**A**) This figure depicts the experimental device 'Procroustis', used for pull-out and torque tests. (**B**) Detail A of the device with modification for the pull-out tests. (**C**) Detail A of the device with modification for the torque tests.

histologic evaluations, rehydrated with decreasing concentrations of ethanol, and treated with two staining techniques: standard hematoxylin and eosin and Masson staining. The Masson trichrome stained mineralized bone in green/turquoise and unmineralized bone (osteoid) in orange/red.

Histologic evaluations were performed using a light microscope (Eclipse TS 100; Nikon, Tokyo, Japan, with X2 and X4 magnification) equipped with a digital camera (Nikon Digital Sight Fi1-L2, Tokyo, Japan) connected to a computer. The histomorphometric data were analyzed using ImageJ software for microscopic images (version 1.51f, National Institutes of Health, Bethesda, MD, USA). Hematoxylin and eosin and Masson trichrome-stained sections were photomicrographed, and 18 to 22 bone areas for each animal were analyzed.

Bone histomorphometry analysis was performed according to the method of Parfitt et al. [40], as updated by Dempster et al. [15]. The following parameters were estimated: the volume of newly formed bone (mm^3) surrounding the implant, the percentage of newly formed bone and the percentage of bone marrow surrounding the implant per optical field, the thickness of newly formed bone (μm), the percentage of mineralized bone in newly formed bone, the volume of mineralized newly formed bone (mm^3) surrounding the implant, and the percentage of mineralized newly formed bone per tissue area.

Quantitative data were pooled to obtain the mean ± SD for each group and reported according to the guidelines of the American Society of Bone and Mineral Research [15]. Specifically, 18 to 22 equidistant stained sections from the central part of the implant site on each tibia were used for histomorphometric analysis. In each section, the surrounding peri-implant area was demarcated at low-power magnification (X2) with the aid of the Nikon digital camera and then the appropriate software, already mentioned, and

photomicrographs were captured. In each photo a $13.653mm^2$ (or 1,228,800 pixels) square was drawn around the empty cavity left by the screw and the peri-implant area, which corresponded to the optical field. The area of compact bone, trabecular bone, and bone marrow in the same square represented the tissue area and were calculated also. First, in this square, we measured the area of compact (or lamellar) bone that was in direct contact with the threads of the screw before its removal, and this area represented the newly formed bone. Its volume was calculated with the following formula: [(area of the peri implant compact bone in each section) X 0.007 mm] and expressed as the volume of newly formed bone (in mm^3) per optical field. The ratio of the area of newly formed bone divided by the area of the square corresponds to the percentage of newly formed bone surrounding the implant per optical field. Peri-implant tissue area apart from newly formed bone consisted of bone marrow and trabecular bone. The area covered by bone marrow adjacent to the newly formed bone and divided by the area of the above mention square, represented the percentage of bone marrow in an optical field. The thickness of newly formed bone (μm) was the width of compact bone, which was in direct contact with the threads of the screw.

In Masson trichrome-stained sections the green/turquoise pixels in the area of the newly formed bone were translated into the mineralized newly formed bone, whereas the red pixels represented the osteoid or unmineralized newly formed bone. Photomicrographs were captured again under x 4 magnification focusing on areas of the newly formed bone and analyzed with ImageJ software. The ratio of the green/turquoise area to the corresponding area of newly formed bone is the percentage of mineralized bone in newly formed bone. This percentage multiplied by the volume of newly formed bone, already calculated above for each section, represented the volume of

mineralized newly formed bone (in mm^3). Similarly, the ratio of the same green/turquoise area to the tissue area represented the percentage of mineralized newly formed bone in tissue area.

Biomarkers Assessment Protocols

Blood samples were obtained by cardiac puncture during the euthanasia procedure [3, 10]. The following oxidative stress biomarkers were evaluated: catalase activity and the concentrations of glutathione and oxidized glutathione were measured in red blood cell lysate, whereas protein carbonyls, malondialdehyde, and the total antioxidant capacity were measured in plasma [32, 61-63]. The hemoglobin concentration of the red blood cell lysate samples was measured using a commercially available kit (HUMAN Diagnostics Worldwide, Wiesbaden, Germany). The total protein concentration of the plasma samples was determined using the Bradford reagent. Each assay was performed in triplicate, and the blood samples were stored in multiple aliquots at -80° C and thawed once before analysis. All reagents were manufactured by Sigma-Aldrich (St. Louis, MO, USA).

In an EDTA tube, 500 µl of whole blood was added, the samples were centrifuged (1370 g for 10 minutes at 4° C), and the blood plasma was collected and used to determine protein carbonyls, malondialdehyde in the form of thiobarbituric acid reactive substances, the total antioxidant capacity, and the total protein concentration. Then, an equal volume of distilled H$_2$O was added to the packed erythrocytes, the samples were centrifuged (4000 g for 15 minutes at 4 °C), and the supernatant, which was the red blood cell lysate, was collected and used to determine catalase activity and the hemoglobin concentration. Next, 100 µl of 15% trichloroacetic acid was added to 100 µl of erythrocyte lysate, and the samples were centrifuged (14,000 g for 3 minutes at room temperature). The clear supernatant was collected and used to measure the reduced glutathione concentration. Immediately after blood collection, 60 µl of N-ethylamaleimide (310 mM in ethanol with a pH of 7.4) was added to 200 µl of whole blood, the samples were centrifuged (1370 g for 10 minutes at 4° C), and the supernatant (plasma) was discarded. Then, an equal volume of distilled H$_2$O was added to the packed erythrocytes, the samples were centrifuged (4000 g for 15 minutes at 4° C), and the supernatant red blood cell lysate was collected. Subsequently, 100 µl of 15% trichloroacetic acid was added to 100 µl of the red blood cell lysate samples and centrifuged (14,000 g for 3 minutes at room temperature). The supernatant was collected and treated with three volumes of dichloromethane (typically 250 µl of supernatant for 750 µl of dichloromethane), shaken in a vortex shaker for 5 minutes at room temperature, and centrifuged (14,000 g for 2 minutes at room temperature). The supernatant was collected and used to evaluate the oxidized glutathione concentration.

Protein Carbonyls

Protein oxidation is a common damaging process after oxidative stress. The plasma protein carbonyls is a relatively stable marker of overall protein oxidation, representing an irreversible form of protein modification [67]. Due to this stability, it is one of the most valuable biomarkers assessing oxidative stress.

A volume of 50 µL of 20% trichloroacetic acid was added to 50 µL of plasma, and this mixture was incubated in an ice bath for 15 minutes and centrifuged (15,000 g for 5 minutes at 4° C). The supernatant was discarded and 500 µL of 10 mM 2,4-dinitrophenylhydrazine (in 2.5 N of hydrogen chloride) for the sample, or 500 µL of 2.5 N of hydrogen chloride for the blank, was added to the pellet. The samples were incubated in the dark at room temperature for 1 hour with intermittent vortexing every 15 minutes, and then they were centrifuged (15,000 g for 5 minutes at 4° C). The supernatant was discarded, 1 mL of 10% trichloroacetic acid was added, and the samples were vortexed and centrifuged (15,000 g for 5 minutes at 4° C). The supernatant was discarded, 1 mL of ethanol-ethyl acetate (1:1 v/v) mixture was added, and the samples were vortexed and centrifuged (15,000 g for 5 minutes at 4° C). This washing step was repeated twice. The supernatant was discarded and 1 mL of 5 M urea (pH = 2.3) was added, vortexed, and incubated at 37° C for 15 minutes. The samples were centrifuged (15,000 g for 5 minutes at 4° C) and the absorbance was monitored at 375 nm. The concentration of 2,4-dinitrophenylhydrazine was calculated based on the millimolar extinction coefficient of malondialdehyde (22 L/mmol/cm) and is presented as nmol/mg of protein [13, 61-63].

Malondialdehyde

Malondialdehyde (MDA) is an endogenous genotoxic product of enzymatic and oxygen radical-induced lipid peroxidation and a potentially important contributor to protein damage and mutation [36]. It is one of the most frequently used indicators of lipid peroxidation [37].

A volume of 100 µL of plasma was mixed with 500 µL of 35% trichloroacetic acid and 500 µL of tris-hydrogen chloride (200 mM, pH = 7.4) and incubated for 10 minutes at room temperature. One milliliter of 2 M Na$_2$SO$_4$ and 55 mM of thiobarbituric acid solution were added and the samples were incubated at 95 °C for 45 minutes. The samples were cooled on ice for 5 minutes and mixed after

1 mL of 70% trichloroacetic acid was added. The samples were centrifuged (15,000 g for 3 minutes) and the absorbance of the supernatant was monitored at 530 nm. The concentration of malondialdehyde was calculated based on the millimolar extinction coefficient of malondialdehyde (156 L/mmol/cm) and is presented as μmol of malondialdehyde/g of protein [14, 61-63].

Reduced Glutathione and Oxidized Glutathione

Glutathione is a water-soluble tripeptide composed of the amino acids glutamine, cysteine, and glycine. Glutathione is an important antioxidant that protects cells from damage due to excessive amounts of reactive O_2 species [58]. Under oxidative stress conditions, glutathione is transformed to oxidized glutathione, and the ratio of glutathione/oxidized gluatathione is altered. The latter is used as an important marker of oxidative stress [69].

A volume of 20 μL of the trichloroacetic acid-treated red blood cell lysate for the samples or 20 μL of dH₂O for the blank was mixed with 660 μL of phosphate buffer (67 mM, pH = 7.95, containing KH_2PO_4 and Na_2HPO_4) and 330 μL of 1 mM 5,5-dithiobis (2-nitrobenzoic acid). The mixture was incubated for 45 minutes in the dark at room temperature and the absorbance was monitored at 412 nm. The concentration of glutathione was calculated based on the millimolar extinction coefficient of 5,5-dithiobis (2-nitrobenzoic acid) (13.6 L/mmol/cm) and is presented as μmol of glutathione/g of hemoglobin [22, 61-63].

A volume of 925 μL of phosphate buffer (200 mM, pH = 7.4, containing KH_2PO_4 and Na_2HPO_4), 5 μL of 20 mM of 5,5-dithiobis (2-nitrobenzoic acid) in phosphate buffer, 20 μL of the N-ethylamaleimide-treated red blood cell lysate, and 20 μL of 4.8 mM nicotinamide adenine dinucleotide phosphate in 0.5% $NaHCO_3$ solution for the samples were added in a cuvette. The blank cuvette contained 20 μL of 15% trichloroacetic acid instead of the red blood cell lysate, and the standard cuvette contained 935 μL of phosphate buffer and 10 μL of 10 mM oxidized glutathione solution (dissolved in fourfold diluted phosphate buffer) instead of the red blood cell lysate. The mixtures were incubated for 15 minutes at room temperature; then, a 20 mU/mL solution of glutathione reductase (dissolved in fourfold diluted phosphate buffer) was rapidly added and the absorbance was monitored at 412 nm for 1 minute. The oxidized glutathione concentration was calculated by subtracting the absorbance change of the blank from the absorbance change of the sample in 1 minute, dividing by the absorbance change of the standard. This value is expressed as nmol of oxidized glutathione/g of hemoglobin [22, 61-63]. The glutathione/oxidized glutathione ratio was also calculated.

Catalase

Catalase is a potent antioxidant enzyme detoxifying H_2O_2. It can be found between blood cells and plasma. Its action renders it a valuable marker of oxidative stress activity [57].

A volume of 4 μL of red blood cell lysate diluted 1/10 in distilled H_2O was added to 2991 μL of phosphate buffer (67 mM, pH = 7.4), and the mixture was incubated for 10 minutes at room temperature. Next, 5 μL of 30% H_2O_2 was added and the absorbance was monitored at 240 nm for 2 minutes. We determined that the catalase activity was based on the molar extinction coefficient of H_2O_2 (40 L/mol/cm), which is expressed as U/mg of hemoglobin. One unit is 1 mmol/min [23, 61-63].

Total Antioxidant Capacity

Total antioxidant capacity evaluates the cumulative action of all the antioxidants present in plasma and body fluids, thus providing an integrated parameter rather than the simple sum of measurable antioxidants. As a result, total antioxidant capacity assesses the synergistic interaction between known and unknown antioxidants, thus providing a valuable insight into the in vivo balance between oxidants and antioxidants [21, 51].

A volume of 20 μL of plasma was added to 480 μL of 10 mM sodium potassium phosphate buffer (pH = 7.4) and 500 μL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl radical, and the samples were incubated in the dark for 30 minutes at room temperature. Then, the samples were centrifuged (20,000 g for 3 minutes) and the absorbance was monitored at 520 nm. The total antioxidant capacity is presented as nmol of 2,2-diphenyl-1-picrylhydrazyl radical reduced/g of protein [51-52, 61-63].

Alpha-tocopherol

A volume of 200 μL of ethanol was added to 100 μL of blood plasma to precipitate proteins, and then 500 μL hexan was added as an extraction solvent. After vortex-mixing and centrifugation (12,000 rpm for 15 minutes), 700 μL of supernatant was dried under nitrogen stream. The dry residues were resuspended in 100 μL of solvent (methanol-water, 95-5 [v/v]), followed by filtration and an injection [9]. All samples were analyzed using the Thermo HPLC Spectra System P4000 (Thermo Scientific, Waltham, MA, USA). Detection and identification were performed using a UV-Visible detector (UV2000, Thermo Scientific). The reversed-phase analytical column, Zorbax XDB-C 18 (2.1 x 50 mm, Agilent, Santa Clara, CA, USA), was operated at ambient temperature. Isocratic elution and

methanol-water 95-5 (v/v) as the mobile phase were applied. The wavelength absorption was 292 nm. The sample injection volume and flow rate were 20 μL and 0.6 mL/min, respectively.

Animal Follow-up

All rats completed the experimental period successfully and uneventfully. There were no tissue healing problems or fractures at the operative sites. The blood samples taken during the euthanasia procedure were adequate for the biochemical evaluations, and all harvested tibias were suitable for biomechanical and histomorphometric evaluations.

The body weights of the alpha-tocopherol-treated animals (326.00 ± 22.30 g) and control animals (332.33 ± 19.81 g) did not differ on Day 0 (95% CI -27.18 to 14.49; p = 0.739). The body weights of animals in both groups increased by the end of the experiment (alpha-tocopherol group: 370.67 ± 17.10 g; controls: 366.00 ± 19.57 g); p < 0.001 in both comparisons. The weight mean difference in Day 0 was -44.67 ± 12.17 g (95% CI -51.41 to -37.93), while at the end of the experiment (Day 29) it was -33.67 ± 10.77 g (95% CI -39.63 to -27.70). No difference was indicated between the weights of the alpha-tocopherol treated rats and those of the control animals on Day 29, with a mean difference of 4.67 g (95% CI -14.23 to 23.64; p = 0.820).

Statistical Analysis

Parametric and non-parametric statistical methods were applied to statistically evaluate the experimental results. Assumptions of normality and homogeneity of variance were tested using the Shapiro-Wilk and Levene's tests, respectively. Sometimes, to evaluate assumptions of normality and stability of variance, we transformed data to common logarithms or square roots. When there was normality and variance in homogeneity, we performed a one-way ANOVA to evaluate the possible major effects of treatment. Differences between the mean values of specific treatments were evaluated using post-hoc multiple comparison tests, namely, Tukey's test and the Duncan multiple range test. Where assumptions about either variability or the form of the population's distribution were seriously violated, with or without transformed data, we evaluated treatment-dependent differences with the Kruskal-Wallis non-parametric test. Differences between the mean values of specific treatments were evaluated using the Wilcoxon rank-sum test (Mann-Whitney U test). To statistically evaluate the weight of the animals, we applied a repeated-measures ANOVA, and we analyzed the interaction between different levels of treatment and the two time-points. For the histomorphometric evaluation, the statistical analysis included the mean and SD. All values were proven to have homogeneity of variance (Levene's test) before two-tailed t tests were performed in the independent groups. All analyses were performed using the statistical software program SPSS version 20.0 (IBM Corp, Armonk, NY, USA). Significance was determined by a p value ≤ 0.05.

We conducted a post-hoc power analysis for the one-way ANOVA using the software package G*Power (Heinrich Heine Univesität, Düsseldorf, Germany) [18, 19] to confirm that the sample sizes would offer adequate statistical power to detect the effect of a specific treatment on the primary endpoints of pullout strength and removal torque of the implants.

Results

Biomechanical Evaluation

The mean value of pullout strength for the alpha-tocopherol group and its SD was 124.9 ± 20.7 newtons (N) versus 88.1 ± 12.7 N in the control group with a mean difference of -36.7 (95% CI bounds vary from -49.6 to -23.9; p < 0.001) (Fig. 4A). The torque median value was 7 (range 5.4 to 8.3) N/cm versus 5.2 (range 3.6 to 6) N/cm (p < 0.001) (Fig. 4B). This suggested better osseointegration of the implants in animals in the alpha-tocopherol group that postoperatively received alpha-tocopherol (Fig. 5).

Histomorphometric Evaluation of Implant Osseointegration

All implants in both groups directly contacted the surrounding bone, with no signs of inflammation at the bone-implant interface. However, better osseointegration was achieved in rats in the alpha-tocopherol group than in the control group (6A-B). The mean volume of newly formed bone surrounding the implant in the alpha-tocopherol group was $29.8 \pm 5.7 \times 10^{-3}$ mm^3 versus $25.3 \pm 7.8 \times 10^{-3}$ mm^3 in the control group (with a mean difference of -4.6, 95% CI bounds vary from -8.3 to -0.8, p = 0.018). Animals receiving alpha-tocopherol had approximately 4.6×10^{-3} mm^3 more newly formed surrounding bone than control rats. The mean percentage of the area of newly formed bone surrounding the implant (alpha-tocopherol group: 31.2% ± 6. 1% versus controls: 26.4% ± 8.2%, with a mean difference -4.8 [95% CI -8.7 to -0.9]; p = 0.018) and the mean percentage of the area of adjacent bone marrow per optical field (alpha-tocopherol group: 27.5% ± 8.6%
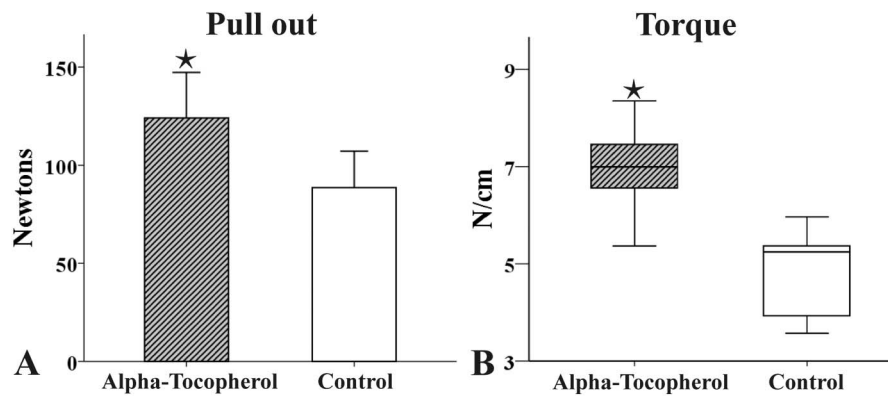
Fig. 4 A-B (**A**) This bar graph illustrates the pullout strength, in Newtons, in control and alpha-tocopherol-treated animals. The bars represent the mean value and the error bars represent the SD. (**B**) This graph illustrates the torque, in N/cm, in control and alpha-tocopherol treated animals. The boxplots represent the median value (50th percentile) and the range of torque (* represents p < 0.05).

versus controls: 20.4% ± 6%, with a mean difference -7.1 [95% CI -11.3 to -2.8]; p = 0.002) were higher in the alpha-tocopherol group than in the control group (Fig. 7A). The mean thickness of the newly formed bone was higher in the alpha-tocopherol group than in the control group (51.9 ± 25.5 μm and 30.7 ± 14.9 μm, respectively, with a mean difference -21.2 [95% CI -25.3 to -17.1]; p < 0.001) (Fig. 7B). The mean percentage of mineralized bone in newly formed bone (alpha-tocopherol group: 74.6% ± 8.7% versus controls: 62.1% ± 9.8%, with a mean difference -12.5 [95% CI -20.2 to -4.8]; p = 0.003) (Fig. 7C) and the

mean volume of the mineralized newly formed bone surrounding the implant (alpha-tocopherol group: 21.6 ± 5.9 X 10^{-3} mm^3 versus controls: 15.7 ± 4.8 X 10^{-3} mm^3, with a mean difference -6.7 [95% CI -9.2 to -4.2]; p ‹ 0.001) were also higher in the alpha-tocopherol group than in the control group. Finally, in the alpha-tocopherol group, the mean percentage of mineralized newly formed bone per tissue area was elevated compared with the control (40.3 ± 8.6% versus 34.8% ± 9%, with a mean difference -5.5 [95% CI -10.4 to -0.6]; p = 0.028) (Fig. 7D).

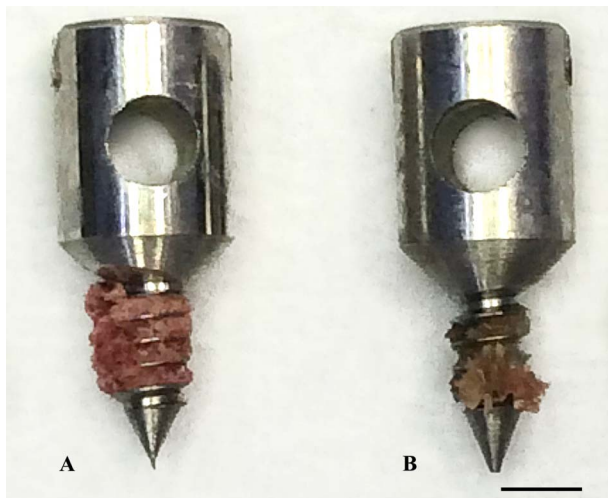### Biochemical Evaluation of Oxidative Biomarkers and Alpha-tocopherol

All studied parameters were different in favor of the alpha-tocopherol group, suggesting decreased postoperative oxidative stress in the animals receiving alpha-tocopherol. The glutathione level in the alpha-tocopherol group was 2 ± 0.4 versus 1.3 ± 0.3 μmol/g of hemoglobin in the control group, with a mean difference of -0.6 (95% CI bounds vary from -0.9 to -0.4, p < 0.001), the median glutathione/oxidized glutathione ratio was 438.8 in the alpha-tocopherol group (range 298 to 553) versus 340.1 (range 212 to 454; p = 0.002) in the control, the mean catalase activity in the alpha-tocopherol group was 155.6 ± 44.6 versus 87.3 ± 25.2 U/mg Hb in the control group, with a mean difference of -68.3 (95% CI -95.4 to -41.2; p < 0.001), the mean malondialdehyde level in the alpha-tocopherol group was 0.07 ± 0.02 versus 0.14 ± 0.03 μmol/g protein in the control, with a mean difference of 0.07 (95% CI 0.05 to 0.09; p < 0.001), the mean protein carbonyl level in the alpha-tocopherol group was 0.16 ± 0.04 versus 0.27 ± 0.08 nmol/mg of protein in the control group, with a mean difference of -0.1 (95% CI 0.05 to 0.15,

Fig. 5 A-B These photographs show pull-out screws in (**A**) specimens treated with alpha-tocopherol and (**B**) control specimens after implantation and mechanical testing (pull-out testing). There is more bone in the threads of the screw from the treated specimen than in the threads of the screw from the control specimen.
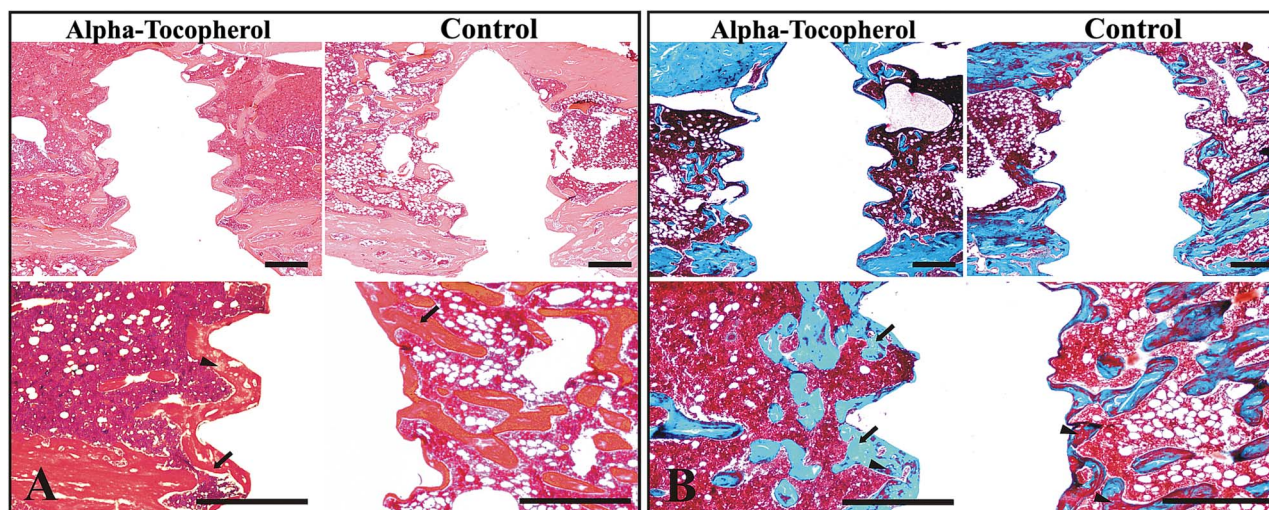
Fig. 6 A-D Photomicrographs show histologic sections of rats in alpha-tocopherol and control groups. The sections were stained with either (**A**) hematoxylin and eosin or (**B**) Masson trichrome stains. (**A**) The rats in the alpha-tocopherol group show more continuous direct bone apposition surrounding the implant and thicker newly formed bone than the control rats. At a higher magnification, the mature bone is presented as a compact structure with a reddish orange color (arrows). The immature bone is displayed as light pink (arrowheads). The nuclei are stained blue. (**B**) Masson trichrome staining allows for identification of mineralized bone tissue by different coloring. Bone structures of mineralized bone are shown in turquoise (arrows), whereas the connective tissue in the newly formed bone (osteoid) is displayed in red (arrowheads). The rats in the alpha-tocopherol group had more mineralized newly formed bone surrounding the implant (turquoise area) and increased cellular density of the bone marrow compared to rats in the control group. (Scale bar = 0.5 mm).

p = 0.002), the mean alpha-tocopherol level was 3.9 ± 4.1 in the alpha-tocopherol group versus 0.9 ± 0.2 mg/dL in the control group, with a mean difference of -3 (95% CI -5.2 to -0.7; p = 0.011), and the mean total antioxidant capacity in the alpha-tocopherol group was 15.9 ± 3.2 versus 13.7 ± 1.7 nmol 2,2-diphenyl-1-picrylhydrazyl radical/g of protein in the control group, with a mean difference of -2.1 (95% CI -4.1 to -0.2; p = 0.008) (Table 1).

Discussion

Alpha-tocopherol, a potent antioxidant agent, seems to exert a positive effect on the fracture healing procedure [17, 34]. Since osseointegration shares common biological pathways with fracture healing [2], this study assessed the ability of alpha-tocopherol to enhance osseointegration of orthopaedic implants as determined by (1) pull-out strength and removal torque and (2) a histomorphological assessment of bone formation. In addition, we examined (3) if there is a correlation between the administration of alpha-tocopherol and the reduction in postoperative oxidative stress that develops after implantation of an orthopaedic implant. Using an in vivo rat model, our results support the research question that postoperatively administered alpha-tocopherol enhances osseointegration of an orthopaedic implant. Although we

found differences in all the biochemical parameters examined in favor of the alpha-tocopherol group, a cause and effect relationship between the administration of alpha-tocopherol and a reduction in postoperative stress, cannot be securely established.

As with similar studies, this one has limitations. One limitation is that an animal model was used [4, 53, 55]. However, the model chosen seemed to be useful, given that multiple evaluation procedures were implemented. Evaluating implant osseointegration immediately postoperatively in clinical studies is extremely difficult and has several limitations, and the long-term evaluation of the same procedure in a clinical setting is time-consuming, costly, necessitates a large number of patients, and can only be used to assess the long-term failure of implant-to-bone bonding (which may be the result of several causes). Although rat bone is similar to human bone [66], the results of experimental studies should be interpreted cautiously and should not be extrapolated to humans without further evaluation.

The existence of many oxidative stress markers renders the choice of markers used in a study evaluating postoperative oxidative stress relatively difficult. However, all markers were selected based on previous studies with reliable results [12, 24, 39, 44, 48]. Furthermore, the combination of all the markers chosen increased the reliability of our evaluations.
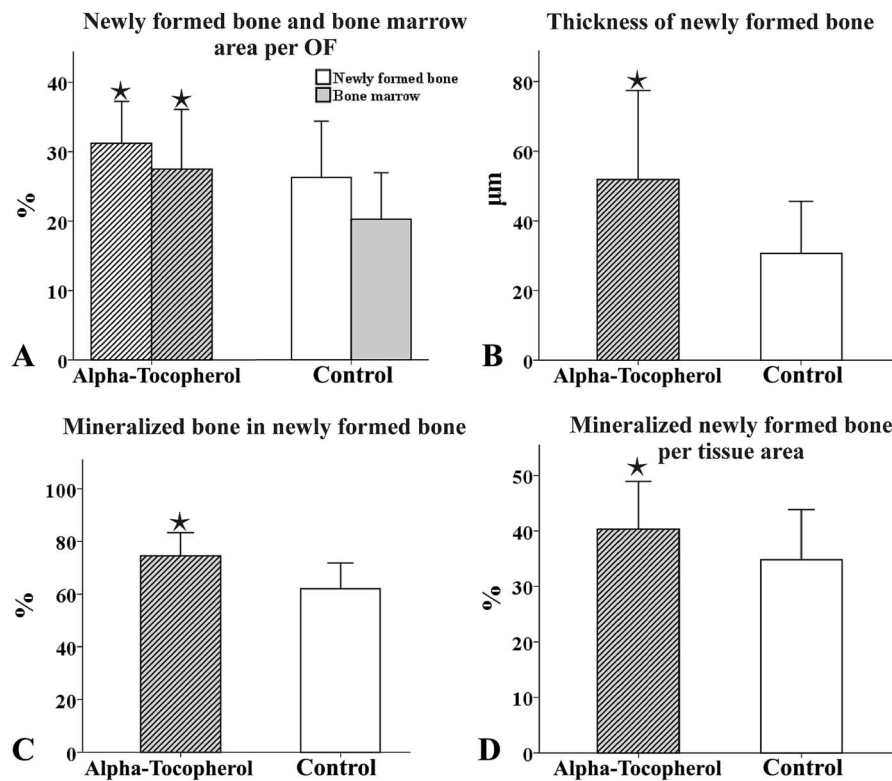
Fig. 7 A-B These bar graphs depict differences in the histomorphometric parameters between the alpha-tocopherol and control groups. The graphs illustrate in (**A**) the percentage area of newly formed bone surrounding the implant and the percentage area of adjacent bone marrow, per optical field (OF), in (**B**) the thickness of newly formed bone (μm) in (**C**) the percentage of mineralized bone to newly formed bone in (**D**) the percentage of mineralized newly formed bone per tissue area in alpha-tocopherol and control groups. The bars represent the mean value and the error bars the SD (*represents $p < 0.05$).

The dosage of alpha-tocopherol was also a limitation. Studies evaluating alpha-tocopherol's anti-oxidative action had nonstandard dosages [4, 16, 27, 29, 30, 34, 64]. Avoiding the extreme high and low dosages of these prior studies, we implemented a dosage of 40 mg/kg of body weight per day. However, even this dose seems extremely high; as translated to the human 40 mg/kg would result in

2-3 g of alpha-tocopherol, when the recommended dosage of alpha-tocopherol for human consumption is about 100 international units (IUs) per day [28]. Apart from the fact that a dosage of up to 1500 IUs per day appears to be safe and even a dosage of 3200 IUs per day does not have a toxic effect in humans [28], an extrapolation of the dosage to the human based on body weight only is not correct. Such an

Table 1. Postoperative oxidative status of animals after alpha-tocopherol administration

Parameter	Control mean (± SD)	Study mean (± SD)	Mean difference (95% CI)	p value
GSH (μmole/g Hb)	1.3 ± 0.3	2 ± 0.4	-0.6 (-0.9 to -0.4)	< 0.001
Catalase (U/mg protein)	87.3 ± 25.2	155.6 ± 44.6	-68.3 (-95.4 to -41.2)	< 0.001
MDA (μmole/g protein)	0.14 ± 0.03	0.07 ± 0.02	0.07 (0.05 to 0.09)	< 0.001
Protein carbonyls (nmole/mg protein)	0.27 ± 0.08	0.16 ± 0.04	-0.1 (0.05 to 0.15)	0.002
TAC (nmole DPPH/g protein)	13.7 ± 1.7	15.9 ± 3.2	-2.1 (-4.1 to -0.18)	0.008
Alpha-tocopherol (mg/dL)	0.9 ± 0.2	3.9 ± 4.1	-3 (-5.2 to -0.7)	0.011
	Median (range)	Median (range)		
GSH/GSSG	340.1 (212-554)	438.8 (298-553)		0.002

GSH = glutathione; GSSH = oxidized glutathione; MDA = malondialdehyde; TAC = total antioxidant capacity.

extrapolation should be based on allometric scaling, with the introduction of a correction factor reflecting the relationship between the body weight and the body surface [35]. Based on these calculations, a dose to humans of 6.49 mg/kg of alpha-tocopherol would reflect the dosage used in this study. This dose is well above the daily requirement for humans but remains still within the nontoxic therapeutic range [28].

Another limitation of our study was the use of only two experimental groups, alpha-tocopherol-treated and saline-treated (control) rats. Though these two groups were sufficient to determine whether alpha-tocopherol enhances osseointegration of steel implants, the lack of a sham group results in an incomplete analysis of whether alpha-tocopherol suppresses postoperative oxidative stress and eliminates any causation between alterations in oxidative stress and implant osseointegration. Further, there was also not a nonsurvival group that would have helped to determine the primary stability of the implant.

The use of 12-week old rats might be considered as another limitation. As the skeletal growth of a rat does not taper off until 7-8 months, a 12-week old rat may be quite young in the musculoskeletal sense. Although the 12-week adulthood is strongly supported by the evidence [11, 42, 47, 65], it is true that at this age the musculoskeletal system is still developing. The time period of the experiment was also determined based on past studies and was considered to be adequate to evaluate the primary stability of an orthopaedic implant [4, 53, 54].

Our primary hypothesis—that the administration of alpha-tocopherol immediately postoperatively and for 28 days after implantation of a stainless steel orthopaedic implant in a rat experimental model would enhance osseointegration of the implant to the bone—seems to be confirmed by our results. This is based on biomechanical and histomorphometric data. Any agent that reduces or eliminates postoperative stress should enhance fracture healing and could positively affect implant osseointegration. Vitamin E is considered the most potent, lipid-soluble, chain-breaking antioxidant in nature [4, 7]. Based on existing animal studies, the effect of vitamin E on fracture healing remains inconclusive [4]. Nonetheless, the effect of alpha-tocopherol on bone formation during the normal bone remodeling phase of secondary fracture healing is promising, although it has not been proven so far to be effective for callous formation [4]. Based on our initial background search for studies on the issue, the most popular proposed mechanism of action for the effect of vitamin E on fracture healing is based on the cellular-protective property of the antioxidant. The protective role of vitamin E was seen in past studies in which alpha-tocopherol in the cell membranes acted as an antioxidant that inhibited lipid peroxidation by scavenging free radicals and breaking the chain reaction [6, 60]. Even vitamin E-coated implants (without systemic administration of vitamin E) showed promising, yet preliminary, results for bone-implant osseointegration [31].

A cause-and-effect relationship between the administration of alpha-tocopherol and a reduction in postoperative stress cannot be securely established by the results of this study alone and certainly requires further confirmation. The effect exerted by oxidative stress on fracture healing remains largely unknown, although it may have a negative impact. After a fracture, oxygen free radicals are produced by both the activation of polymorphonuclear neutrophils in the inflammatory phase of bone fracture healing [4, 49] and by the impairment of the blood supply to the bone's ends [67]. These free radicals inhibit fracture healing [20, 25] by initiating a chain reaction that will cause lipid peroxidation, leading to cell membrane damage and eventually cell lysis [49]. This negative effect exerted by the oxidative stress might also negatively affect osseointegration [33].

To the best of our knowledge, this is the first study examining the ability of alpha-tocopherol to enhance osseointegration of stainless steel orthopaedic implants. By performing a blinded, biomechanical, histomorphometric, and biochemical study, we made every effort to eliminate bias. However, our results should be interpreted with caution and extrapolation of these results to humans should be supported by more research. Further studies with different animal models and/or different implants, studies evaluating the alpha-tocopherol dose response and/or evaluating differences in the sedimentation rate and/or blood coagulation (likely affected by the administration of alpha-tocopherol) should be the next step to securely reach firm conclusions. Large-scale clinical trials confirming the effect exerted by alpha-tocopherol on osseointegration will certainly be needed before moving towards a possible clinical application.

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