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The influence of oral L-arginine on fracture healing: an animal study

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Orale Gabe von L-Arginin beeinflusst die Frakturheilung: eine tierexperimentelle Studie

Zusammenfassung. *Hintergrund:* Die bekannten biologischen Wirkungen von Stickoxid lassen einen Einfluss auf die Knochenbruchheilung erwarten. Ziel der Studie war, zu untersuchen, ob die orale Verabreichung von L-Arginin, einer Ausgangssubstanz von Stickoxid, die Heilung stabilisierter diaphysärer Knochendefekte beschleunigt.

Art der Studie: Verblindeter prospektiver Tierversuch.

Material und Methoden: Wir wählten ein diaphysäres Knochendefektmodell am Meerschweinchen und verabreichten oral hochdosiert L-Arginin. Drei randomisierte Gruppen wurden gebildet: die Kontrollgruppe erhielt nur die Trägersubstanz, die beiden Behandlungsgruppen erhielten L-Arginin über 2 bzw. 4 Wochen ab Operation. Ein 7 mm langer Knochen- und Periostdefekt wurde am Übergang vom proximalem zum mittleren Schaftdrittel gesetzt und mit einem 1,4 mm starken Bohrdraht intramedullär geschient. Nach 4 Wochen wurden die Tiere getötet und beide Femora entnommen. Die radiologische, histologische, histomorphometrische und mechanische Untersuchung erfolgte verblindet.

Ergebnisse: Die Auswertung der Röntgenbilder ergab signifikant mehr Knochenheilungen in den Behandlungsgruppen (2 Wochen: 10/15; 4 Wochen 11/15) im Vergleich zur Kontrollgruppe (3/14). Die mechanische Testserie ergab einen signifikanten Unterschied ($p < 0{,}05$) der notwendigen Energie bis zum Bruch des explantierten Femur zwischen der 4 Wochen-Behandlungsgruppe und der Kontrollgruppe. Histologie und Histomorphometrie zeigten eine signifikante Erhöhung ($p \leq 0.05$) der Integration devaskularisierter Knochenfragmente in den neugebildeten Knochen in beiden Behandlungsgruppen. Veränderungen der unverletzten kontralateralen Femora wurden nicht beobachtet.

Zusammenfassung: Die orale Verabreichung von L-Arginin beschleunigt die Heilung stabilisierter diaphysärer Knochendefekte beim Meerschweinchen ohne merkbare Veränderungen der unverletzten Gegenseite.

Summary. *Background:* The known biological activities of nitric oxide suggest a role in bone healing. We hypothesized that L-arginine, a source of nitric oxide, expedites the healing process of stabilized diaphyseal defects.

Type of study: Prospective blinded animal study.

Methods: Using a guinea-pig model, a 7-mm diaphyseal and periosteal defect was produced in the right femur and splinted intramedullary with a 1.4-mm K-wire. The guinea pigs ($n = 44$) were treated orally in three parallel groups: two treatment groups received high doses of L-arginine (one group for 2 weeks and the other for 4 weeks) and a control group received vehicle only. After four weeks all animals were killed and both femora explanted. Radiological, histological, histomorphometric and mechanical evaluation was performed blinded.

Results: Radiographs showed significantly more healings in the treatment groups (2 weeks: 10/15; 4 weeks: 11/15) than in the control group (3/14). The mechanical energy necessary for femur failure was significantly higher in the 4-week treatment group than in the control group ($p < 0.05$). Histology and histomorphometry showed significantly increased coverage of nonvascularized bone fragments with newly formed bone in the treatment groups ($p \leq 0.05$). The contralateral uninjured femora did not show significant differences between groups.

Conclusions: Oral L-arginine expedites healing in stabilized diaphyseal defects in guinea pigs without detrimentally affecting uninjured counterparts.

Key words: Nitric oxide precursor, animal study, femoral diaphysics, bone defects, bone healing.

Introduction

Bone fractures sometimes take a long time to heal. In difficult cases patients may have to stay in hospital more than six months and undergo several operations. An oral supplementation that expedites healing would have considerable benefits [1], particularly for patients who sustain multiple fractures.

Fracture healing requires a sufficient supply of blood for callus formation. Nitric oxide (NO) is released during blood homeostasis and is believed to have a major role in wound healing and revascularization [2]. NO is also known to inhibit platelet aggregation, leukocyte adhesion, and smooth-muscle cell proliferation [3]. L-Arginine, a well known non-essential amino acid, is metabolized by nitric oxide synthase (NOS) to form NO [4]. Different types of NOS effect bone metabolism but the nature and mechanisms of these effects are unclear [5]. In-vitro application of L-arginine has increased the production of NO and type I collagen in osteoblast cultures from both humans and animals [6, 7]. The systemic application of NO precursors has been found to lead to an increase in bone formation under weight-bearing conditions in femoral fractures in rats, showing NO to be necessary but not sufficient for induction of bone formation [8]. In a growing-rat model, supplementation of L-arginine reduced steroid-induced decreases of bone accumulation [9].

A connection between bone healing, remodeling, growth and the NO cascade has been observed in athletes with amenorrhea. Unlike in postmenopausal women, bone turn-over does not increase in amenorrheic athletes but they do have reduced NO metabolites, spinal osteopenia and low estrogen concentrations. It has been suggested that estrogen exerts its antiresorptive actions on bone via an NO-dependent mechanism [10–12].

We therefore asked whether oral supplementation with L-arginine could have a positive effect on stabilized bone defects under weight-bearing conditions, and expedite healing. To answer this question, we treated diaphyseal defects in a guinea-pig model with oral L-arginine. We assessed bone healing by measuring mechanical parameters and by radiological and histological examination. We also examined the mechanical parameters and X-rays of the uninjured contralateral femora. A guinea-pig model was chosen because this animal has been established as a good model for studies on experimental diaphyseal defects of long bones using intramedullary stabilization [13] We chose a diaphyseal-defect model to reduce the influence on bone healing of contact between the osteotomy sites, in combination with weight bearing [14].

Materials and methods

Animals

Adult male (> 300 g) guinea pigs (Charles River, Sulzfeld, Germany), each with an intramedullary stabilized bone defect, were allocated by computer-generated randomization lists to three groups. Two groups received the following oral treatments at a dose of 100 mg/kg per day: 4-week treatment group (n = 15), L-arginine for four weeks, and 2-week treatment group (n = 15), L-arginine for the first two weeks. A dose of 100 mg/kg was chosen because this is the appropriate dose for small animals [15]. The L-arginine (Merck, Wien, Austria) was dissolved in aqua bidest (9.54 mM). Liquid content was reduced by ultrafiltration, and administration was by pipette to ensure that the animals received the entire specified dose and could drink and feed normally. The third group served as controls (n = 14) and received 0.9% NaCl (Mayrhofer, Linz, Austria). A case record was kept for each animal. The laboratory staff and investigators were blinded to the type of treatment received by the three groups.

Animals were kept one to a cage and identified by fiber-tip pen markings and cage number. They were fed on pellets (Ssniff, Soest, Germany) and given free access to water. The study was reviewed by the institutional board, approved by the Ministry of Science under the provisions of Austrian and European law (GZ 66.009/202) and carried out in accordance with the principles of laboratory animal care.

Manipulation of bone defects

All guinea pigs underwent general anesthesia (sedation with atropin 0.05 mg/kg i.m., and anesthesia with 150 mg/kg ketamine i.m. and 5 mg/kg xylazine i.m.) [16]. A 1.4-mm K-wire was implanted, under sterile conditions, into the medullary canal of the right femur through a small proximal incision. To produce a diaphyseal bone and periosteal defect, the skin on the lateral side of the femur was incised and a 7-mm long segmental resection made in the proximal to middle third of the prestabilized femur shaft with a pair of bone shears. This segmental resection was equivalent to 20% of the entire length of the femur, representing a critical size defect. The wound was then closed. The procedure took about 15 minutes. The contralateral left femur was left intact.

The animals were weighed before the fracture procedure and subsequently on days 7, 14, and 23 and after death. They were killed by injection of an overdose of Vetanarcol (pentobarbital) on day 29 or 30 after fracture. The femora of each animal were removed. After explantation of the intramedullary K-wire from the right femora, both the right and left femora were X-rayed.

All investigators were blinded to the identity of the specimen groups for radiological, histological, histomorphometrical and mechanical analysis.

Radiological evaluation

All specimens were radiologically evaluated. X-rays were taken as far as possible in a plane orthogonally crossing the fracture sites and were evaluated by two independent examiners. Specimens with complete bony bridging were considered to be healed, regardless of whether or not a continuous fracture line was still visible.

Histological examination

Histological sections were obtained using the cutting-grinding technique, which produces excellent histological sections of long bones [17, 18]. Thinner sections of less than 10 µm can be obtained by this method (which is used for thick cortical bones, long bones, jaw bones with teeth containing fillings) than by conventional techniques. However, the quality of the sections is dependent upon the specimen being embedded in the mold at a level at which the cutting edge of the grinder can be directed through the desired plane. To ensure that we obtained useable sections with this high precision procedure, 12 specimens (4 from each group) were selected at

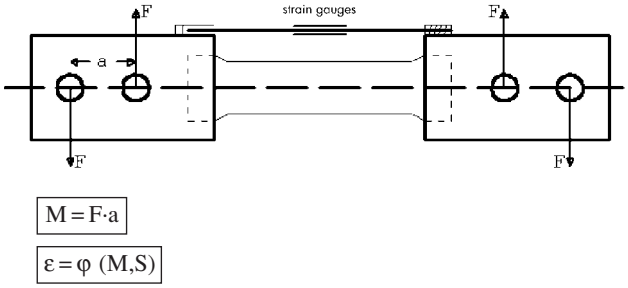

Fig. 1. Sketch of the mechanical apparatus showing the method used (four-point bending test) to distinguish between the mechanical properties of the treated and untreated femora. *a* lever arm; ε strain; *F* applied bending moment; *M* momentum; *S* stiffness of measured device

random for histological examination. These did not undergo mechanical testing to failure. The femora were fixed in 10% neutral buffered formalin, dehydrated with increasing alcohol concentrations, and infiltrated with the resin Technovit 7200 VLC (Kulzer & Co, Friedrichsdorf, Germany). The resin-infiltrated femora were put into resin-filled plastic embedding molds and the resin was then polymerized by blue light. The samples were glued onto plastic slides and positioned at the vacuum plate. The plate was moved towards a saw by a weight in such a way that the specimen was cut in a vertical plane by the diamond blade (band cutting system, EXAKT-Apparatebau, Norderstedt, Germany). The sections were ground to a thinness of 20–25 mm (grinding system, EXAKT-Apparatebau) and stained according to Levai Lazcko [19]. We examined the sections for quality of callus tissue (fibrous tissue, cartilage cells, osteoblasts) and integration or osteoclastic débridement of nonvascularized bone fragments, using an Axioskop microscope (Zeiss, Jena, Germany) at a magnification up to 25 times.

Histomorphometry

For histomorphometric analysis, the specimens were photographed at a resolution of 1 pixel equal to 4.43 µm, using a digital camera (Nikon DXM 1200, Nikon Corporation, Tokyo, Japan) mounted on a microscope (Nikon Microphot-FXA, Nikon Corporation, Tokyo, Japan). To depict the entire longitudinal section of the callus region, 20 individual pictures were assembled automatically with a motorized microscope stage (EK 32, Märzhäuser, Wetzlar-Steindorf, Germany) to form a larger image.

Using an image-processing program (Adobe Photoshop, Adobe, San Jose, USA) false-color images were prepared interactively: pristine bone was coded blue, nonvascularized bone fragments yellow, newly formed bone red, soft tissues white and mineralized cartilage green.

Portions of the perimeter of nonvascularized bone fragments that exhibited signs of resorption (eroded surface) were designated with cyan color; those that showed signs of deposition of new bone were marked magenta.

The region of interest was restricted to a zone 2 mm distally and 2 mm proximally from the former fracture gap.

The areas (mm^2) of all tissue types and the lengths (µm) of nonvascularized bone fragments in contact with their surrounding structures were determined with the morphometry program Lucia G 4.71 (Laboratory Imaging Ltd, Brno, Czech Republic). From these direct measurements the following variables were calculated: percentage of cartilaginous area, percentage of nonvascularized bone fragments, percentage of mineralized tissue (i.e. bone plus mineralized cartilage), percentages of the surface of nonvascularized bone fragments in contact with newly formed bone, and eroded surface of nonvascularized bone fragments.

Measurement of mechanical parameters

The femora from 12 of the 44 animals had been reserved for histological examination and therefore femora from 32 animals were available for measurement of mechanical parameters up to failure. A mechanical apparatus was built to apply a constant bending moment in order to measure bending and stiffness simultaneously in guinea-pig femora (Fig. 1). In order to have comparable loading conditions for all femora, the condyles and femoral necks were embedded into cylindrical molds filled with bone cement. Both femora of each of the 32 animals were evaluated individually by gradually increasing the bending moment up to failure and measuring the accompanying elongation. The energy necessary for failure was calculated. The stiffness (S) measured was that of the embedded femur together with the bending transducer of the measurement device. The data are given as absolute values and as calculated percentages of the individual contralateral uninjured femurs.

For each femur, strain (ε) was measured as a function of the applied bending moment and the initial stiffness was calculated from the linear range of the strain/moment curves. The parameters M_{max} and ε_{max} indicating the end of the linear range and M_f indicating the failure point were the basis for comparison between injured and uninjured femora and were recorded for each femur group for statistical interpretation. The bending moment was always applied with the same loading rate. Stresses and energy were calculated from the measurement of strain using the theory of elasticity [20]. The requirements for appli-

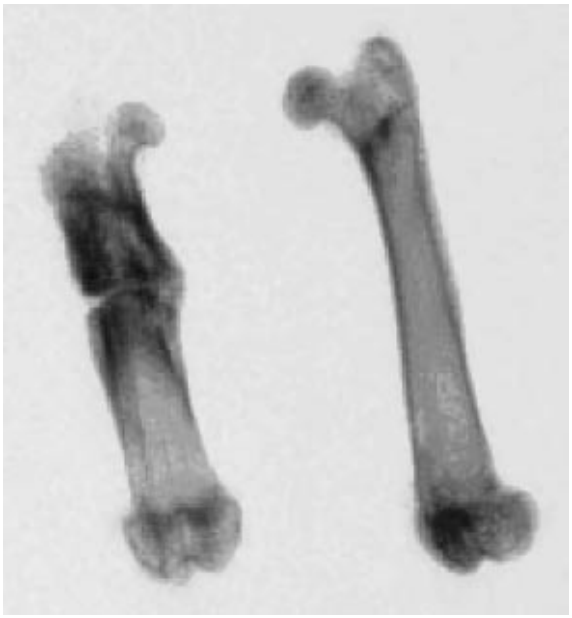

Fig. 2. Radiograph of a pair of femora after removal of the K-wire (guinea pig No. 21, control group). The femur on the left shows incomplete bone bridging, with the fracture line still visible (not healed). The femur on the right is the corresponding uninjured specimen

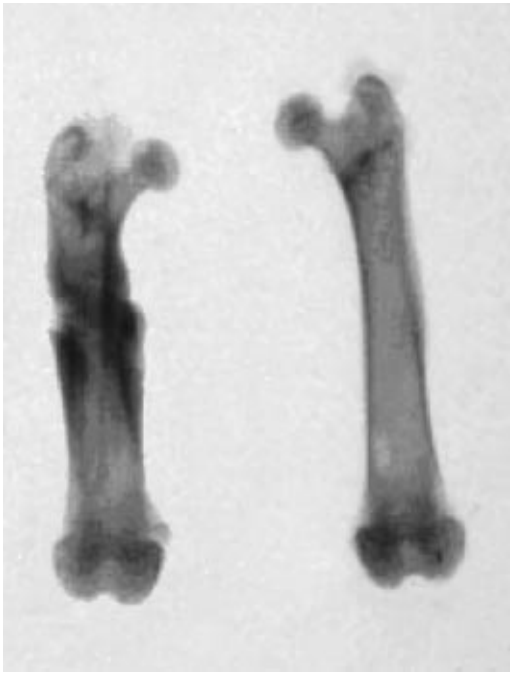

Fig. 3. Radiograph of a pair of femora after removal of the K-wire (guinea pig No. 8, 2-week treatment group). The femur on the left shows bone union and minimal shortening (healed). The femur on the right is the corresponding uninjured specimen

cation of this theory are fulfilled for small long bones, especially if, as in our study, the comparison is made between specimens having the same geometry and texture [21].

Geometrical parameters such as the exact position of the neutral axis and the value of the moment of inertia were not needed, because the aim of the study was to compare healing of diaphyseal defects in treated and untreated femora, and each injured femur was individually compared with the uninjured femur of the same animal. Dimensionless ratios derived from measurements of bending moment, elongation and energy were obtained for each animal. Assuming that the geometry of the two femora was the same in an individual animal, the ratios of the measured data are independent of it and allow a comparison between the groups. The absolute data of the measurements are also presented.

Statistical analysis

The χ^2 test was used for statistical analysis of the radiological evaluation. For analysis of histomorphometry in each of the three groups, the absolute measurements of the area of callus formation, cartilaginous area, area of nonvascularized bone fragments and area of mineralized tissue are reported as median and standard mean error. For analysis of the mechanical measurement in each of the three groups, the absolute measurements of the mechanical parameter, elongation, bending moment and energy are reported as median and standard mean error. Fisher's exact test (SAS software: SAS Institute, Cary, NC, USA) was used for evaluation. A p-value of ≤ 0.05 was considered to indicate statistical significance.

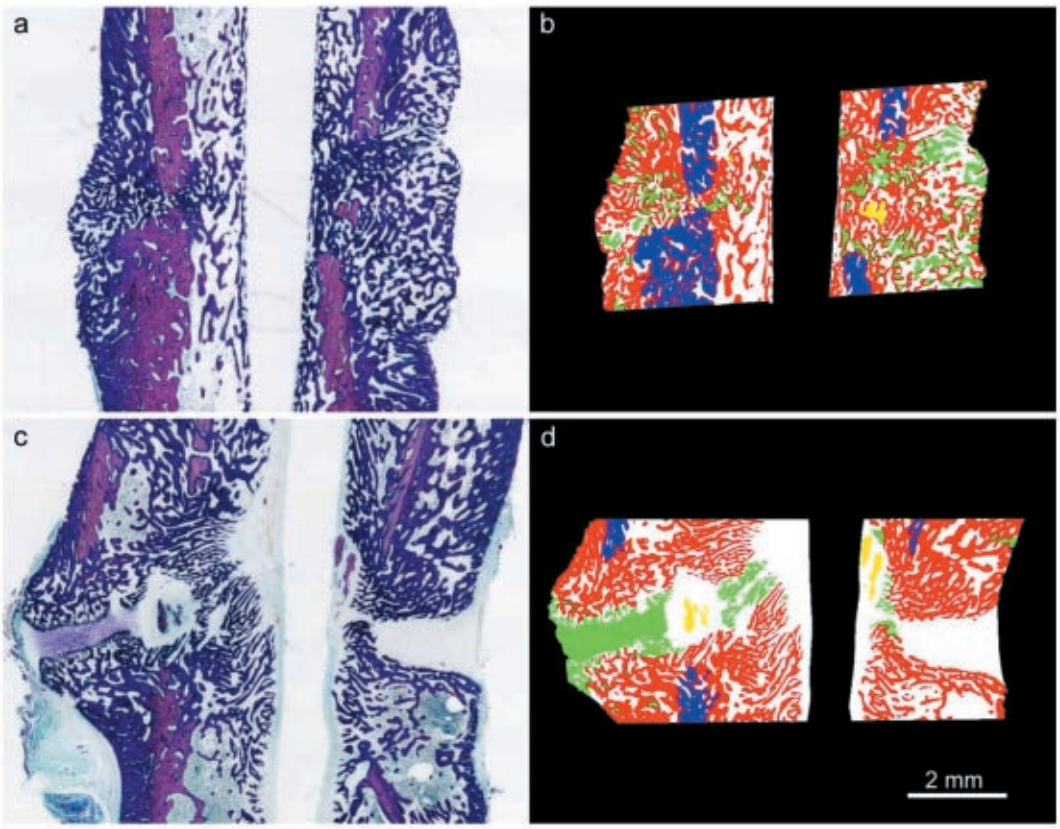

Fig. 4. a–d Histological sections (stained with hematoxylin and eosin) and color-coded specimen for histomorphometry: "old" bone blue, new bone red, cartilage green and bone sequestra yellow. **a, b** Guinea pig No. 34, 4-week treatment group; **c, d** guinea pig No. 47, control group

Table 1. Histomorphometry of the right-side femora with a stabilized critical-size bone defect after four weeks (n = 12, 4 specimens per group); data are given as mean ± SEM

	Callus area (mm^2)	Cartilaginous area (mm^2)	Area of bone sequestra (mm^2)	Mineralized tissue (mm^2)
Control group	31.63 ± 0.86	2.00 ± 0.57	0.11 ± 0.05	16.40 ± 1.46
2-week treatment	26.50 ± 1.70	2.38 ± 0.75	0.09 ± 0.04	14.35 ± 0.94
4-week treatment	25.29 ± 0.77	1.38 ± 0.64	0.07 ± 0.03	14.64 ± 1.23

Results

All animals gained weight and developed normally and therefore qualified for evaluation because general septic complications could be discounted in every case.

Radiological analysis (n = 44)

The two examiners gave the same grades in 90% of the specimens. For the remaining 10% they jointly re-evaluated their findings and agreed upon a grade.

In the control group, bony unions of the defect fracture were seen in 3 of the 14 specimens (Fig. 2). In the treatment groups, bony healing was observed in 10 of the 15 specimens in the 2-week group and in 11 of the 15 specimens in the 4-week group (pooled treatment groups 21/30; Fig. 3). The result of Pearson's χ^2 test was 9.083, indicating a significant difference between the treatment and no-treatment groups for bony healing assessed by radiological analysis (estimated probability value > 99%).

Radiological analyses of the uninjured (left) femora found no visible changes on the radiographs.

Histology of fracture sites (n = 12)

We examined histological sections of the defect fracture sites for quality of bony callus formation in four femora from each of the three groups. In addition, nonvascularized bone fragments near the fracture lines were examined for osteoclastic resorption activity or integration into the bone mass.

In general the same types of cell were seen in all three groups and we saw no differences in the quantity of callus formation. However, there were differences between the treatment groups in the grade of maturity of the callus formation: more cartilage cells were present in specimens from the 2-week treatment group than in those from the 4-week group. We found that nonvascularized bone fragments were integrated into newly formed bone in all but one specimen from each of the treatment groups (Fig. 4a, b), whereas in all the specimens from the control group the nonvascularized bone fragments were not integrated (Fig. 4c, d).

Histomorphometry was used for quantitative analysis of the histological sections. The absolute data of the measured areas are given in Table 1. In both treatment groups the area of callus formation was decreased to about 80% of that of the control group. The percentage of cartilaginous tissue in the callus was 6.5% in the control group, 8.6% in the 2-week treatment group and 5.7% in the 4-week group. The percentage of mineralized tissue (bone and mineralized cartilage) in the callus was 51.6% in the control group, increasing to 54.4% in the 2-week treatment group and 57.9% in the 4-week group. The proportion of cartilaginous to mineralized tissue was 0.126 in the control group compared with 0.159 in the 2-week treatment group and 0.098 in the 4-week group.

The percentage of the surface of nonvascularized bone fragments not covered with (new) bone tissue indicating integration is given in Fig. 5. The difference between the control group and the treatment groups was

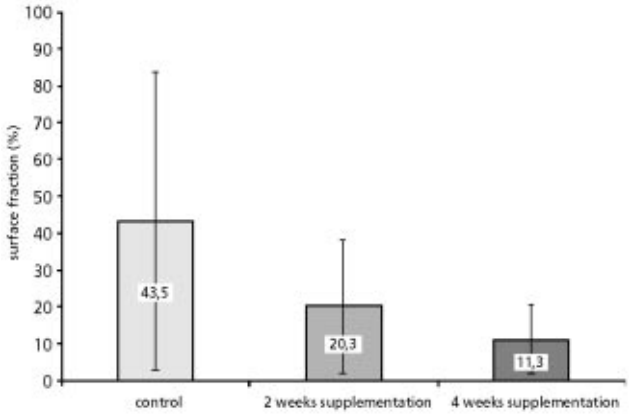

Fig. 5. Histomorphometry: percentage of bone sequestra not covered with bone tissue (mean and SD given; significant difference control vs. treatment groups: $p \leq 0.05$)

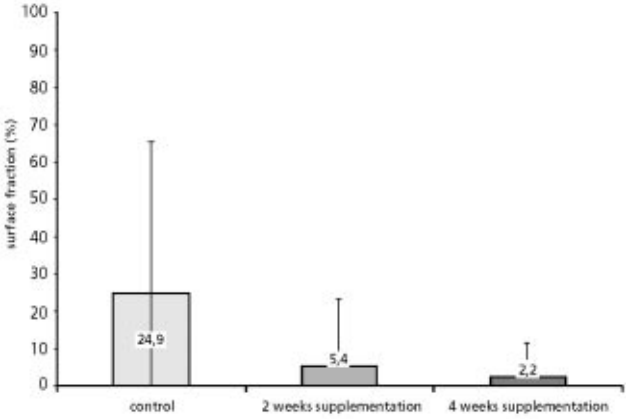

Fig. 6. Histomorphometry: percentage of eroded surface of bone sequestra (mean and SD given; significant difference control vs. treatment groups: $p \leq 0.05$)

Table 2. Mechanical properties of the right-side femora with a stabilized critical-size bone defect after four weeks (n = 32); data are given as mean ± SEM

	Bending (ε) at failure (10^{-3})	Bending moment (M) at failure ($Nm \cdot 10^{-3}$)	Minimum ascent of M-ε relation (Nm)	Maximum ascent of M-ε relation (Nm)	Energy necessary for failure (Nm)
Control group (n = 10)	0.17 ± 0.02	1079 ± 164	7186 ± 1597	7168 ± 1345	0.09 ± 0.01
2-week treatment (n = 11)	0.20 ± 0.03	1335 ± 248	5928 ± 8054	7216 ± 858	0.15 ± 0.05
4-week treatment (n = 11)	0.28 ± 0.05	1610 ± 194	6563 ± 1801	8899 ± 1630	0.18 ± 0.04

significant ($p \leq 0.05$). Figure 6 shows the eroded surface of the nonvascularized bone fragments. The difference between control and treatment was again significant ($p \leq 0.05$).

Mechanical analysis (n = 32)

Mechanical measurements were analyzed with a strain/moment graph. The absolute values are shown in Table 2. The following parameters are given as mean, standard deviation and standard error: elongation at failure, bending moment until failure, bending stiffness (min. and max.) and energy necessary for failure.

The results of the treatment groups were compared with those of the control group (Table 3). Clear trends could be seen in the mechanical properties when comparing the treatment groups with the control group. The increase in energy necessary for failure in the 4-week treatment group compared with the controls was statistically significant ($p < 0.05$). The differences between the two treatment groups were not statistically significant.

Examination of the individual graphs

The curves obtained for all left uninjured femora, regardless of whether the specimens were from the treated or untreated groups, typically showed a curve geometry with a linear gradient up to the point of failure as a basis for calculating the bending stiffness. Typical curves obtained for specimens of right injured femora from the untreated group showed a similar linear gradient up to the point of failure. After the failure point interrupted successive creep occurs. This elasto-plastic behavior is best described by the Bingham model, we therefore refer to it as "Bingham hinge". In contrast, typical curves obtained for specimens of the right injured femora from the treatment groups showed lower almost linear gradients from the outset, whereby a higher point of failure was usually reached than in the untreated injured femora (Fig. 7).

We also compared the left uninjured femora from the treatment groups with those from the control group and found no significant differences between the groups. The following parameters are given as mean and standard mean error: elongation at failure, bending moment until failure, bending stiffness (min. and max.) and energy necessary for failure (Table 4).

Discussion

NO is synthesized from L-arginine, a non-essential amino acid, by at least three isoforms of NOS and is known to function as a vasodilator and neurotransmitter. During homeostasis the endothelium releases a number of vasoactive and platelet-controlling factors including endothelins, prostaglandins, vasoendothelial growth factor (VEGF) and NO [3, 22, 23]. The biological activities of NO, such as inhibition of platelet aggregation, leukocyte adhesion and smooth-muscle cell proliferation, are important for wound healing and might have a role in fracture healing, for which a sufficient supply of blood is also crucial. Furthermore, although the function of NO in bone biology is still unclear, it is known that NO is produced by bone cells and there is evidence that it is an important messenger molecule in bone intercellular communication [5, 8, 24].

Recent work suggests that NO exerts biphasic effects on bone-cell activity [8]. High concentrations of NO impair bone resorption by inhibiting osteoclast formation and the resorptive function of mature osteoclasts [15]. Osteoblast growth and differentiation is also inhibited by high concentrations of NO. Low concentrations of NO,

Table 3. Relation of measured values in the 2-week (n = 11) and 4-week (n = 11) treatment groups with reference to the control group (right-side femora with a stabilized critical-size bone defect after four weeks)

	Elongation at failure/max.	Force at failure/max.	Stiffness min.	Stiffness max.	Energy necessary for failure
2-week treatment	1.15	1.24	0.83	1.01	1.70
4-week treatment	1.61	1.49	0.91	1.24	2.10*

* Significant difference ($p < 0.05$, two sided t-test).

Table 4. Mechanical properties of the contralateral left-side uninjured femora; data given as mean ± SEM

	Bending (ε) at failure (10^{-3})	Bending moment (M) at failure ($Nm \cdot 10^{-3}$)	Minimum ascent of M-ε relation (Nm)	Maximum ascent of M-ε relation (Nm)	Energy necessary for failure (Nm)
Control group	0.25 ± 0.04	1939 ± 200	6708 ± 1028	10329 ± 1292	0.24 ± 0.05
2-week treatment	0.25 ± 0.04	2159 ± 218	7400 ± 915	10882 ± 963	0.27 ± 0.06
4-week treatment	0.27 ± 0.03	2503 ± 225	8334 ± 1216	12375 ± 1217	0.27 ± 0.05

produced by constitutive NOS in bone, might play a part in regulating normal osteoblast growth and the effects of estrogen on bone formation [25].

The ability of L-N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS, to suppress the osteogenic response has been tested in a rat model of mechanically induced osteogenesis. The increase in cancellous bone formation consequent upon mechanical stimulation was completely suppressed when L-NMMA was injected before loading but restored when it was injected after loading, indicating that early release of NO is a key signal in the transduction of mechanical stimuli into subsequent bone formation [26]. Blocking NO in growing rats by oral treatment with aminoguanidine for four weeks induced spinal osteopenia. This effect could be abolished by co-administration with L-arginine [27]. These findings indicate that long-term treatment with aminoguanidine causes an imbalance between bone resorption and bone formation resulting in a decrease in bone mass in growing rats. This suggests that NO produced by inducible NOS is important for the bone-degradation activity of basal osteoclasts in vivo [15]. Rats treated with aminoguanidine also showed a reduced rate of bone formation in the tibial epiphysis; this was completely reversed by L-arginine but these effects were not seen in the tibial diaphysis [28]. In rats the orthotopical application of the NO donor nitroso-bovine serum albumin and the systemic inhibition of inducible NOS by aminoguanidine showed beneficial effects on bone healing [29]. The type-specific and time-dependent expression of NOS isoforms has been suggested as causing the obviously different effects of NOS [30, 31]. The complex mechanism of fracture-healing NOS secretion was also shown in human endothelial cells after application of L-arginine in vitro [32].

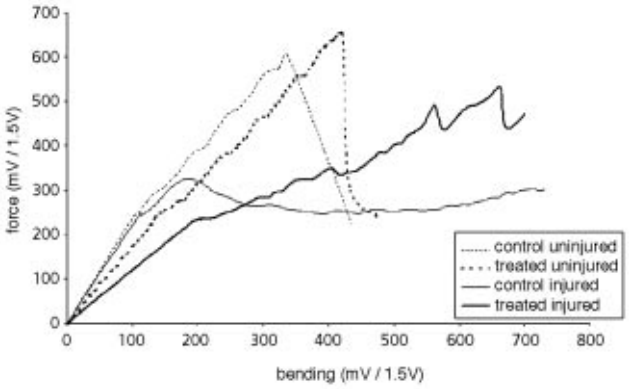

Fig. 7. Example showing typical mechanical testing graphs. Specimen from untreated guinea pig (No. 2) indicated by triangles, from 4-week treated guinea pig (No. 1) indicated by squares (white left uninjured femur, black right injured femur). The graph for the untreated injured femur up to the point of failure shows more or less the same stiffness but no sudden rupture: the failure occurs slowly as shown by the Bingham-Hinge-like curve. The graph for the treated injured femur shows reduced stiffness from the beginning but a higher point of failure. The graphs for the uninjured femora are similar

In our studies we treated a diaphyseal defect in a guinea-pig model with oral L-arginine. We used a bone defect of approximately 20% because this enabled a clearer distinction between specimens from treated and untreated animals than that provided by a simple fracture model [13]. A study in rabbits using a simple fracture model in the tibia, fixed with external fixators, showed differences of only low statistical significance after L-arginine supplementation, which can be explained by the simpler fracture pattern and treatment protocol that allowed easy healing even in the control group [14]. We conducted mechanical tests on the femora and examined radiographs and histological specimens of the femora to assess the effects of the treatment on defect healing. We chose the oral mode of delivery because it is an easy and accessible method. Only adult male animals were used because of the suggestion that estrogen exerts its antiresorptive actions on bone through an NO-dependent mechanism [11, 12].

Mechanical testing showed significant differences in the energy necessary for failure between the 4-week treatment group and the control group. In addition, the differences between these groups for bending moment (up to failure) and elongation failed only marginally to reach statistical significance ($0.066 > p > 0.05$). In almost all parameters, a trend towards improving mechanical properties was seen in both treatment groups and was clearer in the group with four weeks of oral L-arginine.

The initial gradients of the strain-moment graphs in the linear range give the bending stiffness. The non-linear range after the linear range until failure indicated some differences between the groups. However, these ranges overlapped to some degree because of the remarkable scattering. A trend towards improved mechanical properties could be seen in most evaluations, but most importantly statistical significance was reached for the energy data in 4-week treatment vs control. The relevance of this is that the energy necessary for failure is an integral result influenced by all the individual morphological and mechanical properties.

Evaluation of the X-rays in the control group showed bony healing in only three of 14 femora, whereas in the treatment groups the bone was healed in ten of 15 specimens in the 2-week group and 11 of 15 in the 4-week group. Analysis established that the incidence of bone healings of femora in the treatment groups was significantly higher than in the control group (estimated probability >99%). The differences between the two treatment groups were not significant.

Histological examination clearly revealed that the nonvascularized bone fragments (caused by the use of bone shears for segmental resection) integrated into the bony callus formation at the defect sites in both treatment groups. In contrast the nonvascularized fragments in the control group were not integrated. Differences were seen between the treatment groups in bone age and degree of bone maturity, especially in the finding of more cartilage tissue in the specimens from the 2-week treatment group. An explanation for these differences could be an increase in bone formation with inhibition of osteoblast differentiation.

The results of histomorphometry showed a slight decrease in total area of callus in both treatment groups but an increase of the ratio of osseous and cartilaginous tissue within the callus. Compared to the control group the proportion of cartilaginous to osseous tissue was increased two-fold in the 2-week treatment group and 1.3-fold in the 4-week treatment group. These findings correlate with the histological examination and can be explained by an increased maturity of the callus formation in the 4-week treatment group. Significant increase in coverage of nonvascularized fragments with bone tissue was found in histomorphometry between the control and the treatment groups. In addition, the percentage of eroded surface of nonvascularized bone fragments was significantly lower in the treatment groups.

We conclude that L-arginine expedites healing in stabilized diaphyseal defects in guinea pigs, with no detrimental effect on uninjured counterparts. Histological evaluation points to L-arginine improving osteoblast activity and obstructing osteoclasts from disassembling nonvascularized bone fragments at the defect sites. We showed that coverage of nonvascularized fragments with bone tissue is significantly improved after oral treatment with L-arginine and that the eroded surfaces of the nonvascularized fragments are decreased. Radiological evaluation showed significantly better healing of the femoral-shaft defects. Mechanical measurements proved the energy necessary for failure to be significantly higher after four weeks of treatment than in the untreated control group. Other mechanical properties showed improvement but without reaching statistical significance. Studies using larger animals could further lay the foundation for oral treatment with L-arginine as a supplement to current treatment for fractures.

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