Local application of zoledronic acid incorporated in a poly(D,L-lactide)-coated implant accelerates fracture healing in rats

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Background and purpose **Zoledronic acid (ZOL) has been shown in vitro and in vivo to inhibit osteoclastic activity and to regulate osteoblasts. Its antiresorptive effect is used clinically in the treatment of bone-consuming pathologies to prevent skeletal complications. Because of its effect on bone cells, there might be a possible benefit in treatment of fractures by local application from a biodegradable poly(D,L-lactide) (PDLLA) coating of osteosynthetic implants. We analyzed the effect of locally applied ZOL from a PDLLA coating of intramedullary implants on fracture healing.**

Material and methods **Standardized midshaft fractures of the right tibia of 5-month-old rats were stabilized either with uncoated, PDLLA-coated, or ZOL-coated implants. Animals were killed 42 or 84 days after fracture. Tibiae were dissected and mechanically tested.**

Results **Radiographs taken 42 days after fracture showed at least unilateral bridging in all groups. Maximum load and torsional stiffness were highest in the group treated with ZOL. 84 days after fracture, the torsional stiffness of the ZOL-treated group remained higher than that of the uncoated group whereas the maximum load for the control groups reached the results for the ZOL-coated group.**

Interpretation **Local application of ZOL from PDLLA coating appears to accelerate the achievement of mechanical stability in fractures.**

Interest has been focused on administration of stimulating factors to accelerate bone healing. Various substances that have the potential to stimulate osteogenic or chondrogenic cells, or to reduce bone resorption activity in vitro and in vivo have been described (Lind 1998, Baltzer et al. 2000, Schmidmaier et al. 2003).

Bisphosphonates are potent inhibitors of osteoclastic bone resorption (Teitelbaum 2000). They are internalized by osteoclasts and cause apoptosis. Moreover, nitrogen-containing bisphosphonates such as zoledronic acid (ZOL) have been shown, at least in vitro, to reduce osteoclastic resorption activity that does not depend on osteoclast apoptosis (Halasy-Nagy et al. 2001). The explanation for this antiresorptive effect is most likely inhibition of protein prenylation (Roelofs et al. 2006). In addition, clinical studies have shown that biochemical markers of bone turnover are reduced by bisphosphonate treatment (Liberman et al. 1995, Adachi et al. 2001, Emkey et al. 2003). In vitro, bisphosphonates stimulate osteoblastic secretion of osteoprotegerin (OPG) and inhibit formation of receptor activator of nuclear factor-κB ligand (RANKL) (Lacey et al. 1998, Kong et al. 1999, Greiner et al. 2006). The interplay of both factors is responsible for the balance of bone resorption (Hofbauer et al. 1999).

Zoledronic acid (ZOL) is a third-generation nitrogen-containing bisphosphonate that is mainly used clinically in prevention of skeletal complications (e.g. pathological fractures or hypercalcemia of malignancy) caused by malignant tumors (Lipton et al. 2002). Furthermore, recent studies

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have shown its benefit in the treatment of osteoporosis (Black et al. 2007).

Several groups have investigated the influence of bisphosphonate treatment on fracture healing. Most of them showed increased callus volume with improved or unaltered mechanical strength (Goodship et al. 1994, Peter et al. 1996, Li et al. 1999, 2001, Adolphson et al. 2000, Amanat et al. 2005, 2007). Not least because of these favorable results, Fleisch (Fleisch, 2001) stated that fracture repair is not negatively affected by bisphosphonate treatment for osteoporosis.

Previous studies investigating the effect of locally applied ZOL released from a poly(D,L-lactide) (PDLLA) coating on osteoclasts showed a decrease in osteoclast formation and osteoclastic resorption activity (Greiner et al. 2007). The results of the systemic bisphosphonate treatment in fracture healing (Goodship et al. 1994, Peter et al. 1996, Li et al. 1999, 2001, Adolphson et al. 2000, Amanat et al. 2005) together with the results of in vitro experiments investigating the anti-catabolic effect of ZOL released from a PDLLA-coated implant on osteoclasts (Greiner et al. 2007) led us to the hypothesis that local administration of ZOL might accelerate mechanical stability in fracture repair through its effect on osteoclasts.

We therefore investigated the effect of locally applied ZOL on fracture healing in rats.

Materials and methods

Coating technology and substances

Implant coating was performed according to Greiner et al. (2006). Previous implantation and explantation experiments showed a loss of the coating from the implant of less than 5% (Schmidmaier et al. 2001b). 100 mg PDLLA was dissolved in 1.5 mL ethyl acetate at room temperature and the solution was sterile-filtered. ZOL was dissolved in ethyl acetate and was combined with the PDLLA solution to obtain the desired concentration of 50 µM.

Sterile K-wires (1 mm in diameter; Synthes, West Chester, PA) were dipped twice into the coating solution and dried under laminar air flow conditions. The total amount of ZOL in the coating of 1 implant was estimated to about 20 µg.

The following groups were examined: group I, titanium implant alone; group II, implant coated with PDLLA; and group III, implant coated with PDLLA and 2% (w/w) ZOL (ZOL-CI).

Animals and fracture model

Forty-nine 5-month-old Sprague-Dawley rats (250-300 g body weight; Harlan-Winkelmann, Borchen, Germany) were sedated with isoflurane and intraperitoneal anesthesia (ketamine hydrochloride (100 mg/mL, 80 mg/kg body weight) and xylazine 2% (12 mg/kg body weight)). The right tibia and fibula were fractured in a recently described standardized manner (Schmidmaier et al. 2001a, 2004). The leg was placed on two rounded bolts in external rotation. A third bolt was placed 2–4 mm above the tibiofibular junction with a weight of 650 g fixed 15 cm above. An impulse of 1.12 Ns pressure caused a closed transverse fracture of the tibia and fibula in the midshaft. Fractures were reduced, closed, and intramedullary stabilized by coated or uncoated titanium K-wires.

At days 0, 14, 28, 42, and 84, blood was taken from the ophthalmic vein plexus under anesthesia, to evaluate systemic side effects. Routine laboratory parameters (blood count, electrolytes, alkaline phosphatase, glucose, C-reactive protein) were determined with special regard to electrolyte changes and signs of infection. In addition, body temperature was taken rectally and body weight was measured at these time points. Furthermore, wound healing and signs of infection were evaluated and recorded regularly. All experiments were approved by the Animal Experimentation Ethics Committee of Berlin (approval number 0177/04).

Radiographic evaluation

Radiographs were taken with Microvision C mammography film (Sterling Diagnostics, Newark, NJ) on a Mobilett Plus Radiograph Unit (Siemens AG, Erlangen, Germany) in two planes (anteroposterior and lateral view) from the operated limb at days 0, 14, 28, 42, and 84, respectively. Radiographs were examined in blind fashion and evaluation was performed by two independent observers (SG and DB). The inter-rater agreement was 81%. Fractures were classified as being completely bridged, unilaterally bridged, or not bridged (Schmidmaier et al. 2001a), with unilateral bridging including the

union of 1, 2, or 3 out of 4 cortices. The radiographic callus area on digitized radiographs was measured in anteroposterior and transverse diameters using a digital picture analysis program (Image J version 1.240; Microsoft) (accuracy of 0.01 mm). Assuming the callus of the tibia to be an ellipse, the quantity of callus was expressed as the cross-sectional area and compared between the groups. The polar moment of inertia (PMI, in mm^4) was calculated for the callus.

Mechanical testing

Animals from each group were killed at 42 or 84 days after fracture and intramedullary stabilization. Both tibiae were dissected and the outer ends were embedded with bone cement into two embedding molds. A pivoted axis was connected to each embedding mold. A lever, attached to one of the axis loads, changed a linear constant feed rate initiated by a materials-testing machine (Zwick 1455) into a torsional movement. After preloading with an axial force of 5 N, the testing machine applied constant linear propulsion (velocity 2 mm/min) with an equivalent displacement rate of 0.5 degrees per second.

A strain gauge (Fmax = 50 N; HBM, city, Germany) was connected to the free pivoted axis. The torsional force was determined from this gauge and the data were transferred to a computer.

Statistics

The Kruskal-Wallis test was used to identify statistical significance between treatment groups overall. The Mann-Whitney U test was applied to assess the difference between each treatment group, with $p < 0.05$ being considered statistically significant. Data were controlled with Bonferroni correction for multiple comparisons. Statistically significant differences were assumed at the 95% confidence level. Inter-observer agreement for the evaluation of radiographic fracture consolidation was determined with kappa statistics. SPSS software (release 12.0) was used for the statistical evaluations.

Results

Blood and serum analyses, temperature and body weight

No statistically significant changes were found in the blood and serum analyses throughout the experimental period. Serum electrolyte levels were reduced temporarily throughout the investigation period, but did not show changes that could be associated with a systemic effect of ZOL. Leukocytes, C-reactive protein levels, and body temperature were unchanged postoperatively. There was a drop in alkaline phosphatase levels in the ZOL-treated group, but this was not statistically significant (data not shown).

None of the animals examined showed any signs of local or systemic infection throughout the experimental period. Body weight increased similarly in all groups.

Radiographic evaluation

There was a statistically significant increase in radiographic bridging after 42 days in the ZOL-coated group relative to the uncoated group. In the ZOL-coated group (n = 8), all fractures were completely bridged after 84 days. There were no cases of no bridging in any of the 3 groups at 42 days and 84 days (Figure 1 and Table 1).

After 42 days, the callus area was significantly increased in the ZOL-treated group in comparison to the uncoated group and the PDLLA group. The PDLLA coating group showed significantly increased callus area in comparison to the uncoated group also. After 84 days, the mean callus area in the ZOL group remained significantly higher than in the uncoated group and the PDLLA group. There were no significant differences between the two control groups at this time point (Table 2). The increase in polar moment of inertia (PMI) in the ZOL-treated group corresponded to the measured callus area in fracture height. This increase in comparison to the controls was statistically significant (Table 2).

Mechanical testing

42 days after fracture, there was a statistically significantly higher torsional stiffness in the group treated with ZOL than in the PDLLA group and the uncoated group (Figure 2). The maximum load

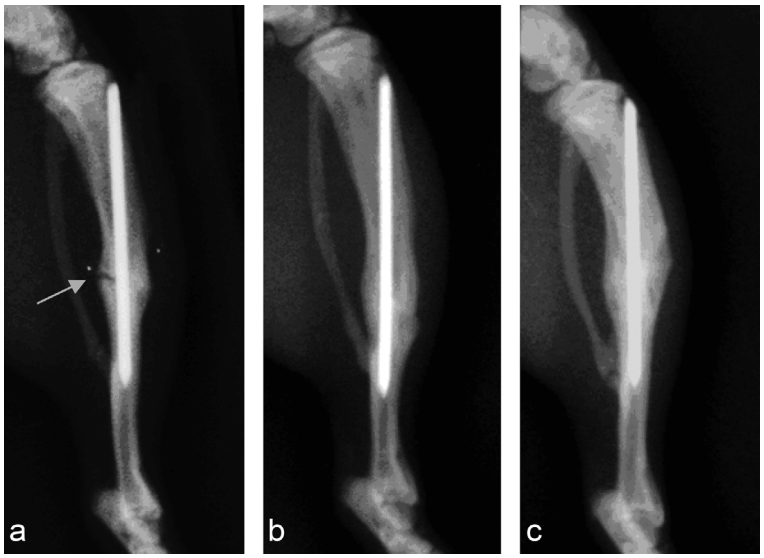

Figure 1. Lateral radiographs of right tibiae of Sprague-Dawley rats 42 days after intramedullary stabilization with uncoated (a), PDLLA-coated (b), and ZOL-coated (c) implants. In 50% of cases, the fracture was incompletely bridged in the uncoated group with a detectable fracture gap (arrow, panel a).

Table 1. Evaluation of callus bridging at the time points investigated

	At 42 days			At 84 days		
Consolidation	Uncoated	PDLLA	ZOL	Uncoated	PDLLA	ZOL
Complete bridging	9	11	15 [a]	8	7	8
Incomplete bridging	8	5	1	1	1	0
No bridging	0	0	0	0	0	0
No. of animals	17	16	16	9	8	8

[a] p = 0.01 vs. uncoated.

Table 2. Polar moment of inertia (PMI) and mean callus area in fracture height at the time points investigated. Values given are mean (SD)

	At 42 days			At 84 days		
	Uncoated	PDLLA	ZOL	Uncoated	PDLLA	ZOL
PMI in cm^4	0.017 (0.019)	0.024 (0.026)	0.041[a] (0.047)	0.001 (0.000)	0.001 (0.001)	0.002 [d] (0.001)
Callus area (cm^2) in fracture height	0.12 (0.017)	0.14 [c] (0.019)	0.18 [b] (0.031)	0.11 (0.012)	0.13 (0.016)	0.16 [e] (0.027)

[a] p = 0.02 vs. uncoated. [b] p < 0.001 vs. uncoated and p = 0.002 vs PDLLA. [c] p = 0.03 vs. uncoated. [d] p = 0.004 vs. uncoated and p = 0.01 vs PDLLA. [e] p = 0.004 vs. uncoated and p = 0.01 vs PDLLA.

after 42 days showed similar results, with significantly higher results in the ZOL-coated group than in the PDLLA and uncoated groups (Table 3 and Figure 2). There were no significant differences in maximum load and torsional stiffness between the uncoated and PDLLA groups.

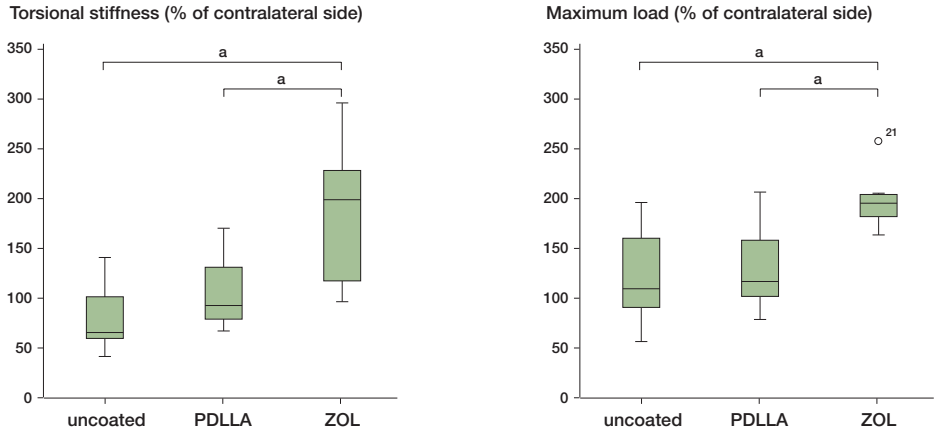

Figure 2. Results of mechanical testing 42 days after fracture, in comparison to the contralateral side (in per cent). The torsional stiffness (a) and maximum load (b) in the group treated with ZOL were significantly higher than in the PDLLA group and the uncoated group. There were no statistically significant differences in maximum load and torsional stiffness between the uncoated group and the PDLLA group.

Table 3. Mechanical testing results for right (fractured) and left (unfractured) tibiae. Values given are mean (SD)

		42 days			84 days	
Group	Uncoated	PDLLA	ZOL	Uncoated	PDLLA	ZOL
n	8	8	8	9	8	8
Right tibia						
maximum load (Nmm)	163 (65)	188 (30)	225 [c, f] (25)	178 (40)	205 (56)	255 [a, g] (45)
stiffness (Nmm/degree)	11 (4)	13 (6)	20 [b] (69	13 (4)	18 (8)	20 (7)
Left tibia						
maximum load (Nmm)	137 (23)	154 [e] (32)	116 (20)	110 (20)	121 (19)	130 [h] (30)
stiffness (Nmm/degree)	15 (6)	13 (4)	11 (3)	12 (5)	13 (5)	12 (4)
% of contralateral side						
maximum load (Nmm)	121 (51)	130 (43)	198 [i] (28)	166 (46)	174 (64)	211 (76)
stiffness (Nmm/degree)	79 (37)	105 (39)	185 [k] (70)	115 (40)	153 (54)	180 [m] (61)

[a] p = 0.004 vs. uncoated. [b] p = 0.009 vs. uncoated and P = 0.04 vs PDLLA. [c] p = 0.05 vs. PDLLA. [d] p = 0.04 vs. uncoated. [e] p = 0.006 vs. ZOL. [f] p = 0.09 vs. control. [g] p = 0.06 vs. PDLLA. [h] p = 0.04 vs uncoated. [i] p = 0.01 vs uncoated and PDLLA. [k] p = 0.006 vs uncoated and p = 0.009 vs PDLLA. [m] p = 0.01 vs uncoated.

The torsional stiffness in the ZOL-treated group 84 days after the operation still remained significantly higher than in the control (Figure 3), whereas the maximum load in the control group and the PDLLA group almost reached the results of the ZOL-coated group, with no significant differences between the groups.

Spearman rank correlation testing revealed a significant correlation between mean callus area and maximum load after 42 days (p = 0.003) and after 84 days (p = 0.002). There was no significant correlation between mean callus area and torsional stiffness at either time point.

Discussion

In previous studies investigating fracture healing, bisphosphonates have been administered systemically either continuously (Nyman et al. 1993, Goodship et al. 1994, Tarvainen et al. 1994, Peter et al. 1996, Adolphson et al. 2000) or in a single

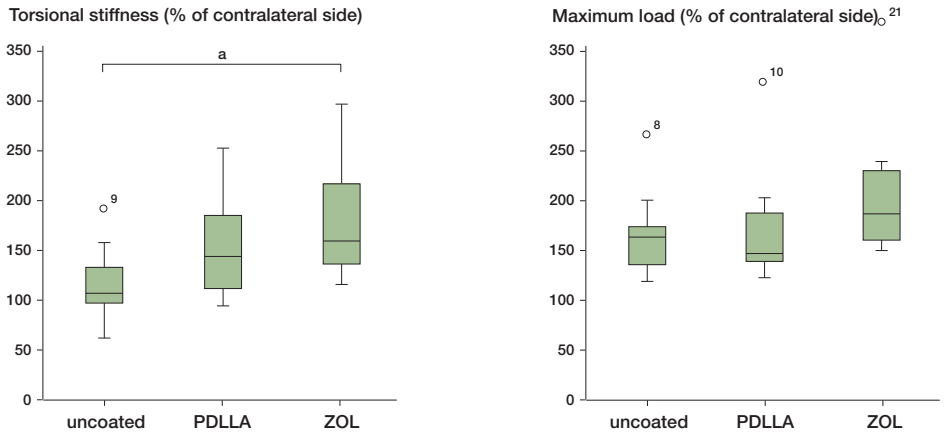

Figure 3. Results of mechanical testing 84 days after fracture, in comparison to the contralateral side (in per cent). The torsional stiffness of the ZOL-treated group remained significantly higher than the controls (a). There were no significant differences in maximum load between the control groups and the ZOL group (b). There were no significant differences in maximum load and torsional stiffness between the uncoated group and the PDLLA group.

dose (Amanat et al. 2005). Recently, local bisphosphonate treatment has been evaluated in vivo, with special emphasis on bone implant integration. Most studies have shown a beneficial effect on osseous implant integration after treatment with local bisphosphonates (Yoshinari et al. 2002, Astrand and Aspenberg 2004, Skoglund et al. 2004, Tengvall et al. 2004, Peter et al. 2005, 2006, Kurth et al. 2005).

We found that ZOL incorporated in an osteosynthetic implant coating improved fracture healing in rats. ZOL was applied locally using an implant coating that is already in clinical use (Schmidmaier et al. 2006). We found no measurable systemic side effects of locally released ZOL regarding renal function, serum electrolytes, or serum calcium. However, there was a drop in alkaline phosphatase levels in the ZOL treated group that was not statistically significant, which has been described as a side effect previously (Black et al. 2007). The radiographic evaluation showed improved bridging of the ZOL-coated group after 42 days in comparison to the controls. The mean callus area was greater in the ZOL-coated group than in the controls after 42 and 84 days, and this was correlated to an increase in polar moment of inertia (Table 2).

Mechanical testing showed increased stability of mid-tibial fractures after treatment with ZOL-CI. Torsional stiffness increased by 134% and maximum load increased by 63% relative to the control, 42 days after fracture. Interestingly, after 84 days mechanical stability of the ZOL group remained almost constant, whereas the maximum load in the control groups increased. There was a further increase in torsional stiffness after 84 days in the control groups. There was a significant correlation between maximum load and mean callus area at both time points investigated, and a corresponding increase in polar moment of inertia. As a result, local administration of ZOL caused production of larger calluses with an increased resistance to loading in the setting used.

We conclude that a delay in remodeling due to the local bisphosphonate treatment may be almost equalized after 84 days. Control and ZOL group showed same values in maximum load at this time point and there was a clear tendency of the control and the PDLLA group to reach the same values of torsional stiffness like the ZOL group. These results suggest that local application of ZOL leads to an acceleration in mechanical stability during fracture repair, most probably due to a temporary delay in remodeling.

Our findings contrast with previously published data showing no significant influence of locally applied pamidronate on mechanical values in a rat osteotomy model (Amanat et al. 2005). As in our study, these data showed increased callus volume and total bone mineral content but in contrast to our data, there was no significant effect

on mechanical stability. Interestingly, Amanat et al. (2005) showed that a single systemic dose of pamidronate significantly enhanced biomechanical stability. Moreover, a slightly increased nonunion rate was found after local application of high doses of pamidronate. Comparison of data remains difficult, however, since there are important differences in the substances used (i.e. pamidronate and zoledronate) and the animal model (open osteotomy as opposed to closed fracture). Moreover, since there has been a substantial effect on stability with systemic doses, the data of Amanat et al. (2005) may indicate that the limited benefit of local application may be due—at least in part—to differences in the doses used. Recently published data have revealed similar results in mechanical testing after a delayed systemic dose of ZOL in a closed fracture model. Interestingly, the delayed exposure was superior to immediate application of ZOL and superior to direct local injection of the substance (Amanat et al. 2007). Administration of the dose under evaluation 2 weeks after fracture showed an increase in maximum load and in torsional stiffness of about 50% in comparison to controls. Even if these results cannot be easily compared because of different settings and fracture models, the present data show comparable results with an increase in maximum load of about 40% and an increase in torsional stiffness of about 80%. Local administration of the substance in the form of direct injection had a limited effect on mechanical properties. The application method we used—comparable to a delayed systemic dose—appears to optimize the effect on mechanical properties.

A previous in vitro study on osteoblasts with ZOL released from a PDLLA coating showed a dose-dependent effect of ZOL. The viability of cells was not affected when they were treated with doses equivalent to up to 100 µM ZOL in coated implants (ZOL-CI), whereas exposure to the pure substance showed a decrease in cell viability and protein synthesis (Greiner et al. 2006). Moreover, evaluation of osteoclast-like cells showed a dose-dependent decrease in the number of TRAP-positive cells and resorption lacunas after exposure to ZOL incorporated in the drug delivery system, or applied as pure substance (Greiner et al. 2007). Even if in vitro results cannot be transferred to the in vivo situation, and effects of bisphosphonates on osteoblasts in vivo have rarely been shown, this proves that there is release of active substance from the coating. Since a concentration of 50 µM ZOL (in the case of total release from the coating) has been shown in in vitro studies to reduce osteoclastic activity without influencing the viability of osteoblast cells, we chose equivalent concentrations of ZOL; 20 µg ZOL was included in the coating, resulting in 2% w/w. This dosage is equivalent to 50 µM ZOL in the PDLLA coating, as used in the cell culture experiments. Even though these in vitro dosages cannot easily be transferred to the situation in vivo, they are the only guidelines available to help find an appropriate in vivo dosage. Although we did not investigate the elution kinetics of ZOL, previous studies using the PDLLA coating have shown that about 50% of the incorporated drug is released within the first 48 h. After this peak, the release curve approximates the maximum release line asymptotically (Schmidmaier et al. 2001b). Comparison of release of ZOL from the coating with the use of the pure substance in vitro showed that exposure to equivalent concentrations of ZOL in the implant coating in comparison to the pure substance had less influence on osteoblastic cell viability at early time points.

In vitro studies showed that exposure to equivalent concentrations of ZOL in the implant coating in comparison to ZOL pure substance had less influence on osteoblastic cell viability at early time points up to 48 h. This difference vanishes at later time points and the same concentrations of ZOL in the coating or as pure substance had similar effects on cell viability (Greiner et al. 2006, 2007). We therefore assume that the kinetics of release of ZOL from the coating may be similar to the earlier mentioned release kinetic study of PDLLA coated implants (Schmidmaier et al. 2001b); thus, early fracture remodeling is accompanied by a potent initial dose of released substance. Afterwards, the concentrations released are lower and remodeling may return to normal.

Several other authors have examined the effects of different bisphosphonates and application methods in fracture healing. Pamidronate given systemically on a weekly basis in sheep pre- and postoperatively resulted in a significant increase in torsional strength in a fracture model (Goodship et al. 1994). An increased callus volume—most prob-

ably due to a delay in remodeling—was observed. Li et al. (1999, 2000), using a rat model, showed the effect of systemically applied icadronate on fracture healing. Histological analysis showed that remodeling returned to control levels after short-term bisphosphonate treatment, and was delayed with continuous treatment throughout the healing phase. These results demonstrate that the benefit of local bisphosphonate treatment is probably due to this temporary delay in callus remodeling, avoiding precocious removal of callus before the fracture fragments are bridged. After diminution of the drug, remodeling recovers to normal and early callus is transformed into mature callus. Early callus formation is characterized by a rapid turnover of tissue. Thus, a potential increase in biomechanical stability of the fractured bone is possible.

Our findings demonstrate that ZOL incorporated in the PDLLA coating accelerates achievement of mechanical stability in fracture healing in a rat model. However, numerous questions still remain to be answered concerning bone remodeling and the influence of bisphosphonates in fracture healing. Analysis of release kinetics and quantitative CT, micro CT and histomorphological evaluation of the calllus should be done.

Contributions of authors

SG did most of the experimental work, analysis and writing, and participated in planning. BW did much of the planning and participated in preparation of the manuscript. DB participated in experimental work and preparation of the manuscript. MA participated in experimental work. PS participated in coating methods. NH was responsible for planning and analysis of the work. GS was responsible for the coating methods and did much of the planning and writing. PA did much of the planning, analysis and writing.

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No competing interests declared

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