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The impact of zoledronic acid on regenerate and native bone after consolidation and removal of the external fixator: An animal model study

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ABSTRACT

We investigated the role of zoledronic acid on the regenerate and native bone after consolidation and removal of the external fixator in a rabbit model of distraction osteogenesis using 28 New Zealand white rabbits.

The rabbits were randomly distributed into two groups. The first group received three doses of zoledronic acid (ZA) 0.1 mg/kg subcutaneously at weekly intervals while the second group received injections of sterile saline. Distraction started on day 7 at a rate of 0.8 mm/day for 12 days.

At week 3 the average lengthening, regenerate density, and regenerate continuity were comparable between the two groups.

At week 11 the regenerate in the treated group had a significant increase in Bone Mineral Density (BMD) and Bone Mineral Content (BMC) compared to the placebo group. On axial compression, the regenerate showed an increase in the peak load and a higher modulus of elasticity in the treated group. At 6 months, radiographs demonstrated signs of osteopenia of the proximal metaphysis in the control group, and failure of new bone formation around the pin sites in the treated group. BMC and BMD value differences between the two groups were not statistically significant. Histologically, there was persistence of more bone trabeculae in the medullary canal of the regenerate with the persistence of the pin-holes in the treated group. Mechanically, the regenerates in the treated group remain stronger in resisting the axial compression. The proximal fragment in the treated group exhibited a statistically significant decrease in the peak load, toughness and efail %. In conclusion, bisphosphonate-treated rabbits have a stronger regenerate during distraction, and directly after removal of the fixator. They do not develop disuse osteopenia in their lengthened tibia. This treatment may shorten the time in the external fixator and prevent fragility fractures in the treated extremity. However, its long-term safety has not yet been established.

Introduction

Distraction osteogenesis (DO) has gained increasing attention over the last three decades since it affords a novel approach to limb lengthening and reconstruction surgery. Wagner method for limb lengthening disappeared with its incapacitating complications from the orthopedist's armamentarium and was replaced by the new concepts of distraction osteogenesis as taught by Dr. Ilizarov [1,2].

DO is based on the principle of Tension-Stress. Gradual traction on living tissues creates stresses that can stimulate and maintain the regeneration and active growth of certain tissue structures [1]. Ilizarov performed most of his experimental work on a canine tibial lengthening model. From this early work, he established principles that were later followed by clinical application [1–3].

The main disadvantage of this technique is that it necessitates an external apparatus on the limb for an average of 1 month/cm of gained length. This period of time is divided into two stages: the first is for lengthening and the second is for bone consolidation. The second stage of this process is the longer of the two.

Different adjuvant treatment approaches have been used in the past to try to enhance bone formation and improve its quality,

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including physical, chemical or hormonal stimulants. However, the results of these studies were equivocal [4–8].

In the 1960s pioneering studies by Fleisch et al. lead to the theory that bone mineralization was regulated by regulating the levels of inorganic pyrophosphates (PPi), the naturally occurring analogue of bisphosphonates [9]. This led to its initial use in the treatment of heritable bone disorders in children [10]. Bisphosphonates were then used to block bone resorption in bony metastases and, more recently, to maintain bone density and reduce fractures in osteoporotic patients [11]. Several animal trials have shown that bisphosphonates can increase the strength and density of the regenerate and reduce the incidence of disuse osteopenia during DO [12–15]. However, these studies did not provide sufficient information about the long-term effect of such treatment on bone remodeling after completion of the lengthening process and removal of the external fixator. Only one study evaluated the long-term impact of bisphosphonates on a rabbit model of DO, and it was found that BP has a transient beneficial effect without preventing long-term bone repair [16]. The aim of this study is to investigate the long-term impact of bisphosphonates on the regenerate and native bone after consolidation and removal of the external fixator.

Materials and methods

Ethical approval for this project was granted through the University Review Board (URB) and the Animal Care Committee at the American University of Beirut (AUB).

Thirty-two New Zealand white rabbits (Harlan, Netherlands) 7 months of age and 1.8–2 kg in weight were gathered for the purpose of this study. Bone Mineral Density (BMD) and Bone Mineral Content (BMC) were measured with dual-energy X-ray absorptiometry (DXA) using a QDR 4500-Hologic machine and lumbar spine software (Bedford, MA).

All rabbits were prepared for surgery in the same manner. They received an intramuscular (IM) injection using a combination of Ketamine 15 mg/kg (Ketalar®, Parke-Davis, Morris Plains, NJ) and Xylazine 4 mg/kg (Gemini®, Rugby Laboratory, Rockville Center, NY). The right leg was shaved and prepped with betadine solution. A longitudinal skin incision over the medial aspect of the right leg was performed. The incision started just distal to the knee joint and measured around 5 cm. The periosteum was incised and a mini lengthener Orthofix M100 fixator (Orthofix, Bologna, Italy) was applied with two proximal and two distal 3 mm pins. The osteotomy was performed 2–3 mm distal to the tibial tuberosity with a power saw. The fibula was cut at the same level with a sharp osteotome. The incision was closed in one layer using Ethibond 3.0 sutures. Local antibiotic solution (ampicillin) was instilled in the wound. Four rabbits fractured their tibia at the time of surgery and were excluded directly. No infections were noted.

After the operation, the rabbits were randomly distributed into two groups (14 rabbits each). The first group received zoledronic acid (ZA) (Zometa, Novartis) 0.1 mg/kg subcutaneously and the second group received a similar volume of sterile saline. The rabbits were kept in a separate room at the animal care facility in single cages and supplied with appropriate feeding. At day 7 the distraction started at a rate of 0.8 mm/day for 12 days. Rabbits in the treated group received two more injections of ZA (0.1 mg/kg) at day 7 and day 14. The rationale behind the usage of relatively high doses of bisphosphonates was previously reported [13].

At the end of the distraction phase, radiographs were performed on all rabbits. Each radiograph was number coded by the research assistant before being interpreted by the musculoskeletal radiologist (N.J.K.) and orthopedic surgeon (S.S.) in a blind fashion. The regenerates were evaluated based on two radiographic criteria: the regenerate continuity and density. These two criteria were graded from 1 to 4 (Table 1).

Table 1
Grading of regenerate continuity and density.

Regenerate continuity	
Grade 1	No radiographic bone formation
Grade 2	Central gap between 2 and 3 mm
Grade 3	Central gap less than 2 mm
Grade 4	No gap
Regenerate density	
Grade 1	No radiographic bone formation
Grade 2	Faint regenerate
Grade 3	Between 3 and 4
Grade 4	Comparable to native bone density

After 11 weeks, all rabbits had their fixators removed and radiographs of the lengthened tibia and DXA studies performed. Four rabbits from each group were sacrificed. The proximal and the regenerate parts of the lengthened tibia, as well as the shaft of the left tibia bone were tested mechanically on a Universal Testing Machine (UTM) measuring the axial compression properties in accordance with ATSM D790M-92. The test was performed at a speed of 2 mm/min. The peak force (F) and the % elongation (efail %) were analyzed directly by the software. The latter provided us with the stress–strain curve. We have calculated the Young's modulus of elasticity as the slope of the linear portion of the curve. The area under the curve (toughness) was also calculated. Axial compression properties were studied over three-point bending since we feel that most fractures, post-removal of the fixators, are due to axial compression upon weight-bearing rather than due to bending.

After the mechanical test, the regenerates were decalcified for 24–48 h and stained with hematoxylin and eosin (H&E) for histologic examination. Two main parameters were studied: cortical thickening and medullarization of the regenerates.

The remaining 20 rabbits were sacrificed at the sixth month. All these rabbits had similar radiographic, bone density, and mechanical studies as those sacrificed at 3 months. Both the proximal fragment and the regenerate of the lengthened tibia were studied histologically.

All gathered data were computed and compared using descriptive statistics or single ANOVA test when applicable.

Results

At baseline (surgery day)

All rabbits were approximately the same age and had similar weights (1.8–2.0 kg). The average BMC was 2.1 (1.6–3) and 2.2 (1.8–2.6) in the treated and the control group, respectively. The average BMD was 0.3 (0.2–0.4) and 0.35 (0.3–0.4) in the treated and the control group, respectively. These results were comparable between the two groups with no statistically significant difference (Table 2).

At week 3

Average lengthening seen on radiographs was 9.1 mm in both groups (range: 8–10 mm). The average score for regenerate

Table 2
BMC and BMD values at baseline and at 11 weeks for both bone and regenerate.

	Pre-op	Bone at 11 weeks	Regenerate at 11 weeks
Mean value of BMC			
Treated group	2.08 g	2.77 g	0.35 g
Control group	2.18 g	2.01 g	0.21 g
P-value	NS	<0.0001	<0.0001
Mean value of BMD			
Treated group	0.33 g/cm^2	0.40 g/cm^2	0.35 g/cm^2
Control group	0.35 g/cm^2	0.30 g/cm^2	0.24 g/cm^2
P-value	NS	<0.0001	0.0003

BMC = Bone mineral content; BMD = Bone mineral density; NS = Not significant.

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continuity was 2.3 (1–3) in the treated group compared to 2.4 (1–3) in the control group. The average score for regenerate density was 2.2 (1–3) in the treated group compared to 2.0 (1–3) in the control group. There was no statistically significant difference between these two groups on all of these parameters.

At week 11

Radiographs were performed on all 28 rabbits. The regenerates had an average anteroposterior (AP) diameter of 8.1 mm (range: 6–11 mm) in the treated group and 7.6 mm (range: 5–10 mm) in the control group. The mediolateral diameter averaged 7.7 mm (range: 7–11 mm) in the treated group and 6.7 mm (range: 5–11 mm) in the control group. These differences did not reach statistical significance. There was a focal gap in at least one cortex in six rabbits; four of these were in the control group. Thirteen rabbits had well formed cortices comparable to the native bone in both AP and lateral views; eight of these were in the treated group.

The DXA studies were also performed at this time. The results showed that the treated group did not have stress-shielding osteopenia of the tibia. The values of BMC and BMD of the whole bone were significantly higher in the treated group. The values of BMC and BMD of the regenerate were significantly higher in the treated group compared to the placebo group (Table 2).

Histologically, there was a mild increase in the cortical thickening in the treated group. However, the most striking difference was the persistence of numerous bone trabeculae in the medullary canal with less lamellar bone and more woven bone in the treated group (Fig. 1).

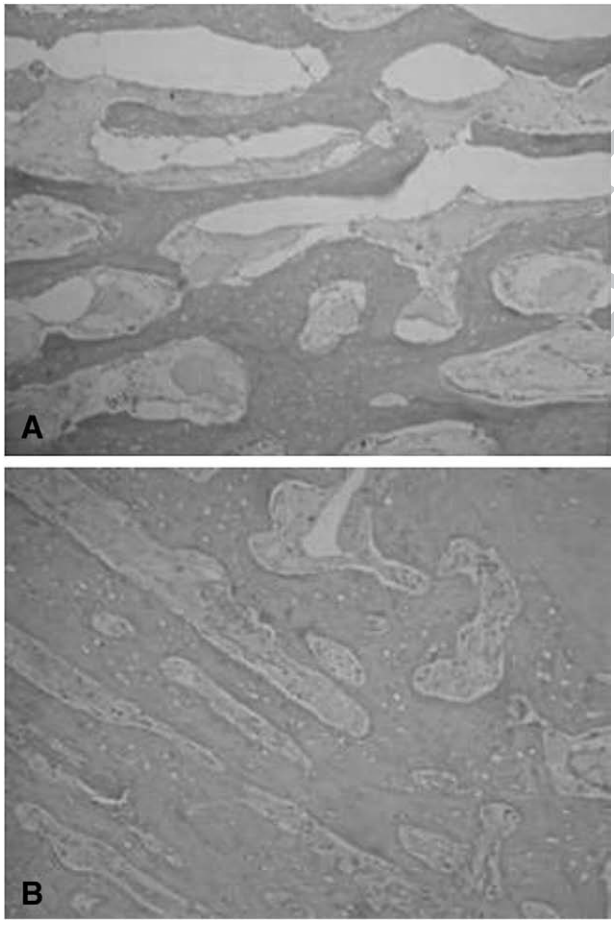

Fig. 1. (A) Histological section of normal bone from a control limb. (B) Histological section from the regenerate bone at 11 weeks. There is increased cortical thickening and increased bone trabeculae in the medullary canal. There is more woven bone and less lamellar bone.

Table 3
Mechanical studies of the regenerate and proximal bone in both groups at 11 weeks.

Parameter	Regenerate (treated)	Regenerate (control)	Proximal (treated)	Proximal (control)
F peak [N]	1643	546	1303	635
E [MPa]	318	59	343	190
Toughness [mJ]	567	237	656	443
E fail %	7.6	8.4	5.4	4.8

F peak = Peak force; N = Newtons; *E* = Young's modulus; MPa = Megapascals; mJ = Millijoules; e fail % = Percent elongation.

Mechanically, both the proximal part of the bone and the regenerate were stronger in resisting the axial compression in the treated group, but the difference was statistically significant only in the regenerate (*P*-value = 0.0086). The treated bone had a higher modulus of elasticity in both segments. Again, the difference was statistically significant only in the regenerate (*P*-value = 0.0024) (Table 3).

At month 6

All rabbits in the control group had decreased radiographic bone density of the metaphyseal area compared to only two rabbits in the treated group. In the control group, there was failure of bone formation in all pins sites in four rabbits and in the proximal pins sites only in one additional rabbit. All rabbits in the treated group have complete obliteration of these sites on radiographs. Only two rabbits in the control group had a persistent well-demarcated regenerate on radiograph compared to seven rabbits in the treated group (Fig. 2).

The DXA studies showed that there was osteopenia in the operated tibia of the control group with BMC and BMD values remaining inferior to the preoperative values. The mean BMC and BMD values of the regenerates in the control group were inferior to those of the treated group. However, these differences were not statistically significant (Table 4).

Histologically, there was persistence of more bone trabeculae in the medullary canal of the regenerate with the persistence of the pin-holes in the proximal fragment in the treated group. These holes were mature with no signs of reactive bone formation or resorption around them (Fig. 3). Foci of woven bone were seen in four rabbits; all from

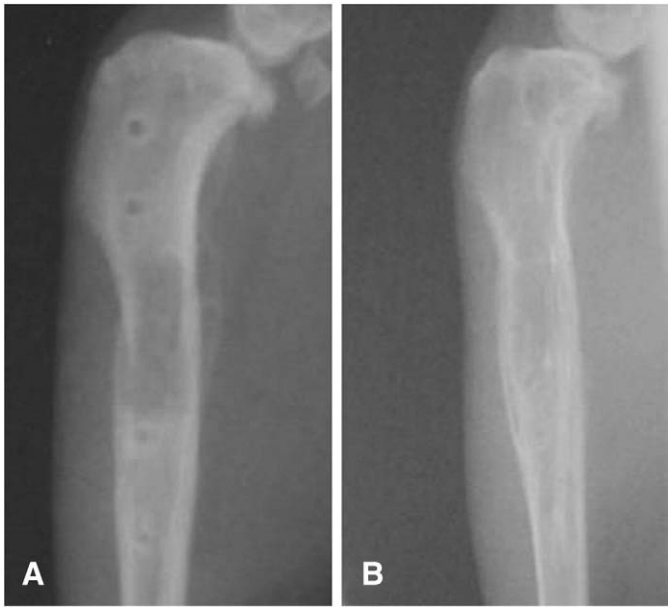

Fig. 2. Plain radiographs showing (A) persistent pin-holes and well-demarcated regenerate in the treated group, and (B) the presence of osteopenia at the metaphyseal region in the control group.

Table 4
BMC and BMD values at baseline and at 6 months for both bone and regenerate.

	Pre-op	Bone at 6 months	Regenerate at 6 months
Mean value of BMC			
Treated group	2.04 g	2.67 g	0.32 g
Control group	2.19 g	2.07 g	0.25 g
P-value	NS	0.0055	NS
Mean value of BMD			
Treated group	0.32 g/cm^2	0.37 g/cm^2	0.32 g/cm^2
Control group	0.34 g/cm^2	0.32 g/cm^2	0.29 g/cm^2
P-value	NS	0.0035	NS

BMC = Bone mineral content; BMD = Bone mineral density; NS = Not sensitive.

the treated group. The cortices of the treated group had an increased thickness as compared to the control group (1 versus 0.7 mm, respectively). The diameter of the regenerate averaged 7.6 mm in the treated group versus 6.64 mm in the control group.

Mechanically, the regenerates in the treated group remain stronger in resisting the axial compression but the difference with the control group became minimal (1417N v/s 1222) (Table 5).

Regenerates in the treated group have a trend toward an increase in Young's modulus, and a decrease in toughness and efail %. However, only the difference in efail% reached statistical significance (*P*-value = 0.028).

The proximal fragment in the treated group exhibited a statistically significant decrease in the peak load, toughness and efail %. In the non-treated tibia, none of these differences reached statistical significance (Table 5).

Discussion

The application of Dr. Ilizarov's principles and methods in limb lengthening has reduced the complication rate and improved the outcome of these procedures. The relatively long time in the fixator and the possibility of fracture occurrence after its removal are the main obstacles in the widespread use of this technique.

The time in the fixator depends on the speed and the quality of new bone formation. Fractures occur secondary to stress-shielding effects of the fixator on the forming regenerate and surrounding bone.

In distraction osteogenesis (DO), osteopenia in the regenerated bone has been attributed to increased bone turnover and subsequently decreased bone formation, leading many authors to focus on pharmacologic agents which could be administered to enhance the regenerated bone in distraction osteogenesis. These agents include a vitamin D derivative (ED-71), recombinant human bone morphogenic protein-2 and 7, growth hormone (GH), thrombin-related peptide (TP 508), calcitonin, and bisphosphonates [4–8,10,12]. All of these play a role in enhancing the formation of bone and increasing its density, but mostly with unclear results. Additionally, a variety of agents have been used to accelerate the anabolic process in distraction osteogenesis, with little success. These were summarized by Tekin et al. and include hormones, growth factors, osteoblast-like cell calcium sulfate, demineralized bone matrix, and ultrasound and electric stimulation [17].

Being a stable derivative of inorganic pyrophosphates (PPi), bisphosphonates have an affinity to the bone mineral, binding to hydroxyapatite crystals [18,19]. They preferentially bind to sites with active bone remodeling and high bone turnover. By doing so, they are able to inhibit the breakdown of hydroxylapatite crystals, thus suppressing bone resorption [19,20].

Recent data suggests that osteopenia in the regenerated bone is due to increased bone resorption [16,21]. Therefore, it seems reasonable to consider administering an anti-catabolic agent such as BP in DO in order to decrease bone remodeling and increase its strength, volume, and density by its direct effect on osteoclastic activity [11]. Indeed, this has been shown recently in several animal models [12–14,21,22].

Although not directly studied, the healing time can be expected to be shorter when at any specific time, the regenerate demonstrates increase strength and bone density as shown in several animal models [12–14,22].

The data from our study has confirmed the previously reported impact of ZA on lengthened bone [14,16,22]. These studies have found that with ZA there was increased load capacity [14], increased strength, increased BMC and BMD values, and reduced disuse atrophy, especially with the redosed group [22,23]. A long-term study using ZA found that there was an increased in bone mass with a transient increase in callus strength, without any negative effects on bone repair in the long-term [16]. Short-term studies of local alendronate infusion found similar results, including improved mechanical strength, morphological properties, and increased BMD in the regenerate [15,24]. Another study using intravenous pamidronate also reported increased mechanical strength in addition to improved BMD and BMC [13]. A similar study using the same bisphosphonate had similar findings in addition to reduced disuse osteoporosis in the treated group [12]. In a small human trial using intravenous pamidronate or ZA in DO patients with poor regenerate quality, it was found than local BMD improved in six out of seven patients treated [25].

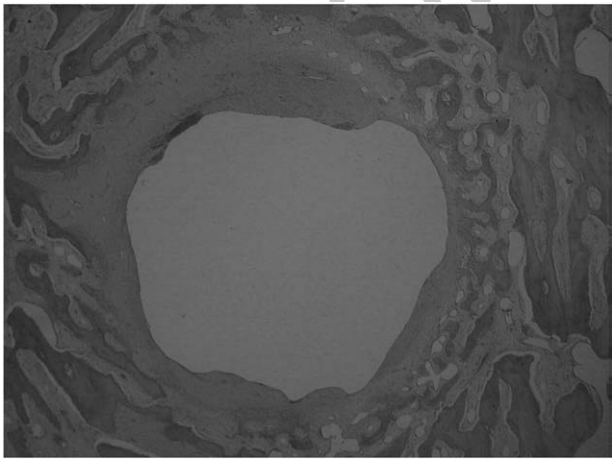

Fig. 3. Histological section of a pin-hole showing mature bone with no signs of reactive bone formation or resorption around it.

Table 5
Mechanical studies of the left limb, regenerate, and proximal bone in both groups at 6 months.

	Control	Treated	*P*-value
Left limb			
F peak [N]	719	694	NS
E [MPa]	902	899	NS
Toughness [mJ]	490	468	NS
E fail %	1.1	1.7	NS
Regenerate			
F peak [N]	1222	1417	NS
E [MPa]	327	522	NS
Toughness [mJ]	1182	758	NS
E fail %	11.3	5.8	0.019
Proximal			
F peak [N]	918	686	0.017
E [MPa]	164	178	NS
Toughness [mJ]	773	374	0.004
E fail %	7.5	5	0.044

F peak = Peak force; N = Newtons; *E* = Young's modulus; MPa = Megapascals; mJ = Millijoules; e fail % = Percent elongation; NS = Not significant.

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In our study, there was preservation of the bone mineral and prevention of disuse osteopenia of the lengthened tibia in the treated group as compared to the control group. This effect was sustained throughout the experiment and the difference between the two groups was maintained at the sixth month. This finding is in agreement with clinical reports which show that osteoporosis can be seen on radiographs even up to one year after external fixator removal [26].

A short-term study of the mechanical properties of ZA on bone in a rabbit model of DO suggested that the lengthened bones were significantly stronger in the ZA-treated animals, but were not significantly stiffer. However, the energy absorbed to failure (efail %) and failure strain were not significantly reduced in either group, suggesting that bone does not have an increased risk of brittle fracture as a result of ZA therapy [14]. Our study showed almost identical mechanical findings at 11 weeks, confirming these results.

Clinically, these findings will have two consequences: the first is the prevention of disuse atrophy while fixators are in place and just after its removal. This may decrease the incidence of fractures. The second consequence is that the stronger the regenerate is in resisting axial loading, the earlier the dynamization of the frame which can enhance bone healing and contribute to the prevention of disuse osteopenia. Earlier consolidation of the regenerate will translate into earlier removal of the external fixator and a better healing index.

A study by Little et al. showed that in rabbits given pamidronate there was increased cortical bone formation around the pin-hole sites of the external fixator, yet they did not study the mechanical properties of the regenerate [12]. Another experimental study showed that there was an increase in BMD values at the pin-hole regions in animals treated with ZA [23]. In our study we also found that pin-hole sites were maintained in the proximal segments of the treated group, though it was not clear if ZA had any effect on bone resorption or formation around these sites. Yet in mechanical studies we showed that these segments were significantly weaker and more brittle when compared to the untreated group, despite the absence of osteopenia. Therefore, we inferred that these weaker segments were due to persistent pin-hole sites. Further studies, perhaps with three-point bending tests, may be helpful in demonstrating that the persistence of these sites is the cause of increased weakness of the proximal segments.

A long-term study on the effect of ZA on a rabbit model of DO showed that the delayed remodeling of the regenerate, leading to an increase in bone mass, was transient with similar results between the treated and control animals after 44 weeks [16]. Our experiment confirmed these findings since although there was an increase in radiographic ossification, mineral content and strength of the regenerate throughout the experiment, the discrepancies in the regenerate properties seemed to be only significant at the eleventh week. The differences between the two groups were less significant at the sixth month.

Histological studies of the effect of ZA on bone in DO found that there was increased new bone formation in the treated group [17,27]. Also, osteoblasts were significantly increased in the treated group of one study, which the authors hypothesized was caused by ZA, leading to increased bone formation [27]. Another study, which compared alendronate to calcitonin in a rabbit model of DO, found that in the alendronate-treated group there was significantly more trabecular bone formation relative to the calcitonin-treated group, and increased osteoblastic activity relative to the control group [28]. In our study we found that, at 6 months, there was persistent increase in bone trabeculae in the treated regenerate, with increased cortical thickness.

All these findings at 6 months raise questions with regards to remodeling of the regenerate and whole bone. For the first time, there are subtle findings to incriminate bisphosphonate as a leading cause for brittle bone. The increase in Young's modulus and a decrease in toughness and efail% at 6 months, delineate a trend toward more brittle bone in the treated regenerate and in the proximal metaphysis as well.

In this study, the doses of ZA used in this study are superior to the clinical doses used in osteoporosis or in oncology. The rationale of these megadoses was to increase the likelihood of finding a significant discrepancy between the groups. It is possible that our three doses regimen was the cause behind the brittle bone.

In summary, bisphosphonate-treated rabbits have a stronger regenerate during distraction and after removal of the fixator. They do not develop stress-shielding osteopenia in their lengthened tibia. This treatment may shorten the time in the external fixator and prevent fragility fractures in the treated extremity after removal of the fixator. The long-term benefits are questionable. The persistence of pin-sites, delay in medullarization of the lengthened column, loss of mineral content from the regenerate and bone between the third and sixth month, decrease in strength of the proximal fragment at sixth month, and the trend towards more brittle bone in the treated group are not reassuring for the promotion of the clinical use of these drugs in bone lengthening.

The long-term safety of the use of ZA in bone lengthening and the recovery of a normal bone remodeling pattern merits further investigation.

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