

Effect of heparin-sodium and enoxaparin on rats tibial fracture healing: clinical, anatomopathological, and biomechanical approach

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SUMMARY

An experimental study in rats was accomplished to evaluate the effect of anticoagulant on fracture union, according to clinical, anatomopathological, and biomechanical approaches. Manually, after bone perforation, fracture was produced in the diaphysis of the right tibia, and maintained without immobilization in 72 male rats of Wistar lineage, each 60 days in age with a medium weight of 242 grams. Twelve hours after the fracture, anticoagulant treatment was initiated, and maintained for 28 days. One group received subcutaneous heparin-sodium in a dose of 200 UI/kg every 12 hours, while another group received enoxaparin in a dose of 1mg/kg every 12 hours, doses preconized for treatment of thromboembolism in humans. The third group, the control, received distilled water. During the experiment, the animals were clinically evaluated and after 28 days, sacrificed. In the animals of the three groups, the clinical evolution was simi-

lar. By means of anatomopathological analysis made by descriptive and quantitative study, the presence of fibrosis, cartilage, and bone was homogeneous among in the three groups, always with a prevalence of osseous tissue. The biomechanical study, accomplished through a flexion test, demonstrated a stiffness rate and maximum load similar in the three groups. No clinical, anatomopathological, or biomechanical differences were found, resulting in consolidation of all the fractures in agreement with the adopted approaches, concluding that the heparin-sodium and the enoxaparin in these doses, method, and time of administration used did not interfere with the consolidation of tibia fracture in the rat.

Keywords: Tibia fractures; Wound healing; Rats, Inbred strains; Heparin; Low molecular weight heparin, Biomechanics; Histology.

INTRODUCTION

Anticoagulant agents have been increasingly used as a therapeutic measure or prophylactic treatment for thromboembolic disease in patients with fractures⁽⁶⁾. When one takes into account how frequently anticoagulants are given to patients with fractures, one question arises: whether anticoagulants can adversely affect bone repair or not.

Trueta⁽¹⁹⁾ and Buckwalter & Cruess⁽³⁾ stated that anticoagulants delay bone consolidation in classic texts of orthopedic and traumatology literature. Other authors also confirmed this hypothesis^(1,4,10,12,16,18,20). Other studies, however, did not find any effect of anticoagulants upon bone fracture repair^(2,5,13,14,17).

Taking into account the controversies found in literature, the present study was designed to clinically, histologically, and biomechanically evaluate the effect of heparin sodium and enoxaparin upon bone consolidation by using an investigational model of open tibial fracture in rats. This study was approved by the Ethics Committee for Animal Research of the Medicine School of Botucatu, Unesp.

MATERIAL AND METHOD

The present study evaluated 72 Wistar male rats aged 60 days with a mean weight of 242 g, provided by the Central Vivarium of the UNEASP - Botucatu Campus. Six groups were formed by draw: A₁ and A₂, controls given distilled water; B₁ e B₂ were treated with enoxaparin; and C₁ and C₂ were treated with heparin sodium. The histological study was carried out in three groups of 8 animals each (A₁, B₁ and C₁) while the biomechanical study was carried out in the remaining three groups of 16 animals each (A₂, B₂ and C₂). After the adaptation period of 7 days, an open fracture was produced in tibial diaphysis. Following disinfection and using an aseptic technique a tibial midpoint hole was initially drilled with a 1-mm bit, the fracture was then manually completed. No type of immobilization was used.

The drugs under study were subcutaneously given 12 h following fracture and at 12-h intervals for 28 days. Group-A animals were given 0.1 ml/kg/day of distilled water; group-B animals were given 2 mg/kg/day of enoxaparin; and group-C animals received 400 IU/kg/day of heparin sodium. Blood-clotting

Study carried out at the Department of Surgery and Orthopedics (DCO) of the Medicine School of Botucatu (FMB) - Unesp

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tests were not carried out because the expected changes induced by these drugs, given at these doses, are well known in our laboratory and widely described by the model standardization data available in literature and in others studies carried out at our laboratory^(6,9).

Twenty-eight days following fracture, the animals were killed with a lethal dose of pentobarbital and the right tibia was removed. Clinical, histological, and biomechanical analyses were carried out. The histological characteristics of bone calluses were described according to the amount of different tissues (fibrosis, cartilage, and bone); dimension of the bone callus; alignment of primary bone ends; presence of necrosis at the site of fracture and primary bone ends; characteristics of bone marrow (primary and newly formed); presence of inflammation (nonspecific and granulomatous of the foreign body type).

The amount of the different types of tissue was categorized based upon a scale ranging from 0 to 3 (0 = absent; 1 = small amount; 2 = moderate amount; 3 = great amount). Results were analyzed by the non-parametrical Friedman and Kruskal-Wallis tests.

The biochemical analysis included the three-point flexion assay with the universal assay machine of the electromechanical type of the Experimental Laboratory of the Department of Surgery and Orthopedics of the Medicine School of Botucatu - UNESP. Load was applied until the rupture of specimen under study. Preload was not used. The mechanical assay was carried out according to the following standardization: load cell: 5000 N; course: compression; load application speed: $30 \times 10^{-9} \text{ m/min}$; scales: 3000N (ordinates) and $2.5 \text{ m } 10^{-3}$ (abscissas) (Figures 1 a-b).

RESULTS

Clinical Considerations

No difference was found in the behavior of animals in the three groups. Animals did not develop infection or hematomas at the site of drug administration.

Twenty-seven days following surgery one rat from the control group died of unknown cause. Three animals were ruled out from the investigation: one animal from the control group due to death; one from the group B (enoxaparin) and another animal from the group C (heparin sodium) due to bone fracture during dissection.

Histological Analysis

As subgroup A₁ (controls), the bone callus was characterized by bone tissue in predominantly all animals and in general in a great amount; islands of cartilaginous tissue in five animals: in a small amount in two and in a moderate amount in three animals; areas of fibrosis in seven animals, in a small amount in the majority of them; cartilaginous tissue was concomitantly found in five animals.

Mature or maturing bone tissue has been shown in general, with endochondral ossification characteristics, osteoblastic border, and rare foci of osteoclasts. Newly formed bone was mainly found on the periphery of the bone callus and is continued by the periosteum of the primary bone; it was also found in a smaller amount in the central area. Proliferation of bone tissue was always more marked, when primary bone ends were not juxta-

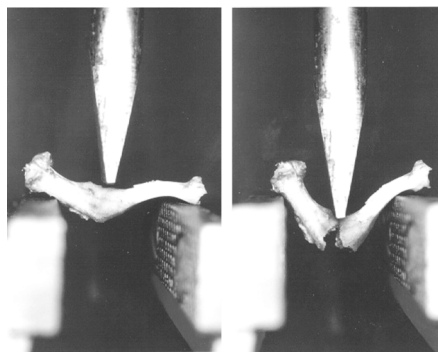


Figure 1 a-b - Flexion assay until rupture of the specimen under study

posed at the site of the fracture. In these cases, the size of the bone callus was also greater.

Cartilaginous tissue was mature when present. No preferential location of cartilaginous tissue was identified in the bone callus.

In the seven cases with fibrosis, there were five where necrotic bone fragments and fibrose were associated and five cases where fibrosis and granuloma of the foreign body type induced by animal hair.

Within the bone callus newly formed normocellular bone marrow was also found while the bone marrow of the primary bone was always hypercellular. Several foci of

hemorrhage were found in both bone marrows, and macrophages with hemosiderin pigments were found in some animals.

In other animals, viable primary bone ends were seen. They were continuous to newly formed bone. Nonspecific inflammation was not seen in any case (Figure 2).

In the bone callus in enoxaparin-treated animals bone tissue predominate; cartilaginous tissue was present in only two animals (in a small amount in one and in a moderate amount in the other one); fibrosis was seen in five animals predominantly in a small amount.

This subgroup had characteristics similar to those found in controls, such as predominance of newly formed bone in the bone callus with preferential peripheral proliferation and presence of endochondral ossification. The amount of newly formed bone tissue was correlated with the size of the bone callus with the following results: small amount in two, moderate amount in three, and great amount in three. The size of bone callus in enoxaparin-treated animals was generally smaller than that of controls.

The mature cartilaginous tissue was randomly distributed within the bone callus and was seen only in two animals, a lower number than that found in the control group.

Fibrosis was found in five cases; in three of them, it was related to animal hair-induced granuloma, horny lamellae, necrotic tissue, and hemosiderin. In one animal, fibrosis was associated with minimal necrotic bone fragments. Both the morphology of hypercellular and newly formed (normocellular) bone marrow was shown to be similar to those found in controls, with hemorrhagic foci.

In all animals, viable primary bone ends were seen. They were continuous to newly formed bone. Nonspecific inflammation was not seen in any case (Figure 3).

As for heparin-treated animals, newly formed bone predominated in bone callus in all animals and was generally found in a moderate amount; cartilaginous tissue was found in four animals (in a small amount in one and in a moderate amount in three of them); fibrosis was seen in seven animals (in a minimal or small amount in six and in a moderate amount in one of them).

As compared to the two subgroups mentioned earlier, the predominance of bone tissue, endochondral characteristics, and mainly peripheral location were found to be similar. However, in the animals of this subgroup, usually the amount of newly formed bone was smaller than those found in controls and also in enoxaparin-treated animals. As for the size of bone callus, a correlation was always found between the amount of newly for-

med bone tissue and bone end alignment, a finding consistent with that found in the previous subgroups. The majority of heparin-treated animals had smaller bone callus than those of control and enoxaparin-treated groups.

The maturation and random distribution of cartilaginous tissue in the bone callus were similar to those found in other subgroups. Cartilaginous tissue was found in four animals: in a small amount in one and in a moderate amount in three of them, a finding consistent with that found in control animals (5). However, that number was greater than that found in enoxaparin-treated groups (two cases).

Usually fibrosis was related to necrotic bone fragments (four cases) or animal hair-induced granulomas, horny lamellae, and necrotic material (four cases), a finding similar to that found in other subgroups.

The characteristics of preexistent (hypercellular) and newly formed (normocellular) bone marrow were similar to those found in the animals of the previous subgroups, both having hemorrhage foci.

Viable primary bone ends and lack of nonspecific inflammation were found in all animals (Figure 4).

The quantitative evaluation of the different types of tissue found in the bone callus from animals included in the histological analysis (subgroups A₁, B₁, C₁) is shown in Table 1).

Biomechanical Analysis

The weight data of the tibias used in the biomechanical assay (subgroups A2, B2, C2) are shown in Table 2. The maximal load data and the stiffness rates for subgroups A2, B2 e C2 are shown in Tables 3 and 4, respectively.

DISCUSSION

The doses of heparin sodium and enoxaparin used in the present study are those recommended for the treatment of thromboembolism in humans⁽¹⁵⁾. The administration of anticoagulant agents was initiated 12 hours after fracture and maintained for 28 days. Therefore, anticoagulants were present in almost all phases of bone consolidation, except for bone remodelling.

The animals were killed after a period of time considered appropriate for bone consolidation, according to Müller⁽¹¹⁾.

Clinical Analysis

The uniform behavior of the animals of the three subgroups as well as the fact that the involved tibia bore load in all animals within approximately one week following fracture with a similar clinical course allows one to conclude that anticoagulants did not clinically interfere in early phases of bone consolidation.

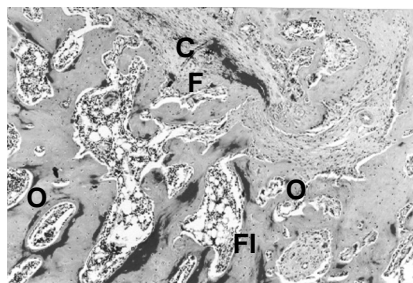


Figure 2 - Bone tissue (O) in a great amount with endochondral ossification. Cartilaginous tissue (C) in a moderate amount. Fibrous tissue in a moderate amount, mature (F) and immature (FI) - (Alcian Ponceau, 25x - AO).

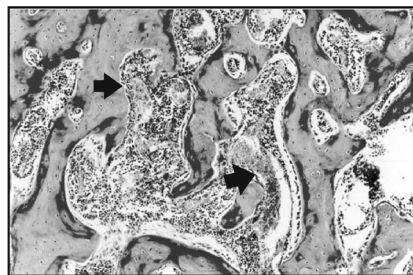


Figure 3 - Newly formed bone tissue with endochondral ossification. Normocellular bone marrow with hemorrhagic foci. (→) - (Alcian Ponceau, 33x - AO).

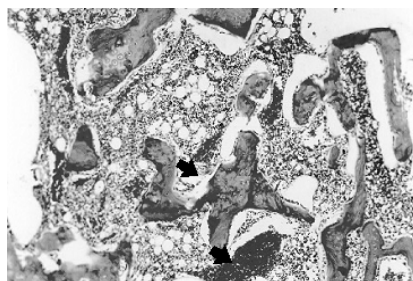


Figure 4 - Newly formed bone tissue with endochondral ossification. Bone marrow with normocellular hemorrhagic foci (→) - (HE, 25x - AO).

Histological Analysis

Ham⁽⁷⁾ identified three phases of bone consolidation: granulation, proliferation, and remodelling. The three phases can coexist with predominance of one over the other, depending on the moment of consolidation.

The first phase, granulation, is characterized by hematoma formation and presence of inflammatory cells, including polymorphonuclear leukocytes, macrophages, and lymphocytes. When the inflammatory response subsides, the necrotic tissue and exudate are reabsorbed and fibroblasts appear⁽³⁾. Disorders during this phase can interfere in bone consolidation and include insufficient formation of fibrin clot⁽¹⁸⁾, more persistent presence of hematoma⁽⁸⁾, hemorrhage at the site of the fracture⁽¹⁾, formation of an extensive hematoma resulting in vascularization deficit⁽¹⁶⁾, and more liquid consistency of hematomas⁽¹⁹⁾, all of them induced by anticoagulants.

However, Flatmark⁽⁵⁾ did not find any abnormality in the formation of fibrin in anticoagulant-treated animals. He also found that the size of hematoma did not interfere in bone consolidation. Nosny et al.⁽¹³⁾ induced the lysis of the clot between bone fragments and did not find abnormalities in bone consolidation.

The second phase, proliferation, is characterized by the marked presence of pluripotent mesenchymal cells at the site of the fracture. According to the majority of authors, anticoagulants can induce changes in the proliferative phase of bone consolidation. Stinchfield et al.⁽¹⁸⁾ found a decreased number in bone matrix cells; Minola et al.⁽¹⁰⁾ found central fibrosis and a longer cartilaginous phase; Aulisa⁽¹⁾ observed delayed formation of new bone, greater callus, and intenser presence of cartilage; Rokkanen & Slätis⁽¹⁶⁾ found longer time of callus maturation, with greater formation of cartilage; Flatmark⁽⁵⁾ described abundant formation of cartilaginous tissue; Dodds et al.⁽⁴⁾ showed delayed ossification of callus and fracture; Nilsson & Granström⁽¹²⁾ observed increased chondroid reaction; Wikesjo et al.⁽²⁰⁾ found a smaller amount of repair tissue.

The excessive formation of cartilaginous tissue was attributed to an abnormal repair⁽¹⁰⁾, greater size of the hematoma or to "some intrinsic property" of the anticoagulant⁽¹⁾ and poor vascularization⁽¹⁶⁾. Flatmark⁽⁵⁾ and Nilsson & Granström⁽¹²⁾ did not explain the phenomenon.

In the present experiment, animals were killed four weeks following fracture induction. Predominance of chondroid tissue suggesting a longer cartilaginous phase was not found. Fibrosis, cartilage, and bone were simultaneously found in all subgroups, with predominance of bone tissue.

Group	Tissue			Friedman's Test and comment
	Fibrosis (F)	Cartilage (C)	Bone (O)	
Control - A ₁	1 (0 - 2)	1 (0 - 2)	3 (2 - 3)	12.00 (p<0.01) (F ≧ C) < O
Enoxaparin - B ₁	1 (0 - 2)	0 (0 - 2)	2 (1 - 3)	12.56 (p<0.01) (F ≧ C) < O
Sodium Heparin - C ₁	1 (0 - 2)	0.5 (0 - 2)	2 (2 - 3)	9.43 (p<0.01) (F ≧ C) < O
Kruskal - Wallis's Test and comment	1.07 (p>0.05) A ₁ ≧ B ₁ ≧ C ₁	2.24 (p>0.05) A ₁ ≧ B ₁ ≧ C ₁	4.23 (p>0.05) A ₁ ≧ B ₁ ≧ C ₁	

Table 1 - Median, scores (minimum and maximum) of the tissue amount found in the bone callus and results of comparisons of subgroups and tissue types - subgroups A₁, B₁, C₁.

Group	Mean and Standard Deviation	
Control - A ₂	0.954 ± 0.089	
Enoxaparin - B ₂	0.936 ± 0.097	
Sodium Heparin - C ₂	0.932 ± 0.089	
Hypotheses	Results of the test and p-value	Comments
Similarity of mean profiles	F = 0.30 (p>0.05)	The mean profiles of subgroups are similar
Group Effect	F = 0.81 (p>0.05)	A ₂ ≧ B ₂ ≧ C ₂

Table 2 - Mean and standard deviation of the parameter tibia weight (g) of the animals from the three experimental groups, tested hypotheses, calculated statistics, and comments.

Group	Maximal Load	
Control - A ₂	65.47 ± 17.53	
Enoxaparin - B ₂	72.91 ± 19.24	
Heparin sodium - C ₂	65.69 ± 16.30	
Hypotheses	Test Results and p value	Comments
Similarity of mean profiles	F = 1.28 (p>0.05)	The mean profiles of subgroups are similar
Subgroup effects	F = 0.64 (p>0.05)	A ₂ ≧ B ₂ ≧ C ₂

Table 3: Mean and standard deviation of the variable maximal load (N) obtained in the three study groups, hypotheses under study, calculated statistics, and comments.

Group	Rigidity Coefficient	
Control - A ₂	59.7 ± 6.9	
Enoxaparin - B ₂	59.3 ± 10.5	
Heparin sodium - C ₂	55.2 ± 12.9	
Hypotheses	Test Results and p-value	Comments
Similarity of mean profiles	F = 0.02 (p>0.05)	The mean profiles of subgroups are similar
Subgroup effects	F = 1.60 (p>0.05)	A ₂ ≧ B ₂ ≧ C ₂

Table 4 - Mean and standard deviation of the variable rigidity coefficient (N/10⁻³ m) obtained in the three study groups, hypotheses under study, calculated statistics, and comments

A method similar to Müller's⁽¹¹⁾ was adopted so as to confirm the observations of the descriptive study by quantifying the callus tissues. The subjective impression of the histological description was then translated into numbers.

The statistical analysis of results showed that predominant bone tissue was found in all subgroups and the amount of fibrous, cartilaginous, and bone tissues was similar when subgroups were compared.

No difference was histologically found in the tissue composition of the bone callus with evidence of bone consolidation, a finding consistent with those reported in literature, except for Stinchfield et al.⁽¹⁹⁾ and Dodds et al.⁽⁴⁾.

Stinchfield et al.⁽¹⁸⁾ and Dodds et al.⁽⁴⁾ used spongy bone in their experiments, in contrast with the majority of studies mentioned above and the present study itself where cortical bones were used. Since spongy bones have a greater surface area, vascularity, and cellularity than cortical bones⁽³⁾, maybe they are more susceptible to the anticoagulant action during bone consolidation.

Biomechanical Analysis

The animals used in the present study were well matched for age, sex, body weight, treatment being the only variable among the study groups. The weight of fractured tibias used in the biomechanical analysis was similar in all three subgroups, thus evidencing that the treatment did not interfere in the bone weight. Differences, if any, found in the biomechanical tests were not to be due to bone weight.

The speed of 30 mm/min is considered a middle speed. Assays carried out with middle or low speeds offer increased safety, allowing the progressive accommodation of the specimen under study, supported without any fixation since the load itself attaches the specimen during the assay.

The structure-related mechanical properties were evaluated in the present study, including the maximal load required for rupture and the stiffness rate (the constant K).

The maximal load value is indicated by the assay report and corresponds to the limit of material resistance before rupture. The value of the stiffness rate is calculated with the help of the load-deformation diagram, also provided by the assay report.

The maximal load data and the stiffness rate are complementary. The analysis based upon only one of these properties can lead to a misleading conclusion that different materials had the same mechanical properties since the same force can be required to break materials of different elasticity or vice versa. The association of maximal load values and stiffness rate allows one to more accurately evaluate the mechanical properties of the material.

Flatmark⁽⁵⁾ was the only author to carry out a biomechanical study as a criterium of evaluation of the anticoagulant effect upon bone repair. By using the traction assay he evaluated radial fracture in rabbits in different phases of the experiment and observed that bone rigidity was gradually increased in anticoagulant-treated animals up to six weeks following fracture when it became similar to that observed in the control group.

In the present experiment, the analyzed mechanical properties were similar, thus indicating that the anticoagulant therapy did not affect bone consolidation, as shown by the biomechanical study.

CONCLUSION

With the methodology used and the analysis of the results obtained in clinical, histological, and biomechanical evaluations, the present study showed that the administration of enoxaparin and heparin sodium did not interfere in bone consolidation in rats.

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