

THE EFFECTS OF LOW-MOLECULAR-WEIGHT HEPARIN (ENOXAPARIN) ON BONY CALLUS FORMATION IN RATS' FEMURS – AN EXPERIMENTAL STUDY

SALIM MUSSI FILHO¹, LEANDRO VIDIGAL², RODRIGO ABBUD CANOVA², HENRIQUE ABREU DA CRUZ², FRANCISCO JOSÉ ZANILO³, LUIZ ROBERTO GOMES VIALLE⁴;

SUMMARY

Venous thromboembolism is a serious complication that may follow fractures. The most commonly used anticoagulant treatment is low-molecular-weight heparin (LMWH). There are some studies showing that this drug may interfere on bone metabolism. With the objective of evaluating the LMWH influence on the process of bony callus formation, we conducted an experimental study on rats. Sample was constituted of 22 Wistar male rats, which were submitted to diaphyseal fracture on their right femurs. They were divided into two groups of 11 subjects each. In the control group, the animals received saline solution and in the study group, they

received LMWH – enoxaparin – in a daily basis, during 28 days. After that period, the rats were submitted to euthanasia for femur assessment purposes. At the macroscopic study, union was verified in 11 animals (100%) not receiving enoxaparin, and in 10 animals (90.9%) receiving the study drug. At the histological study, the formation of bony callus was verified in all femurs. It was concluded by this experiment that enoxaparin does not cause changes on the bone union process in Wistar rats' femurs.

Keywords: Venous Thrombosis; Fractures; Heparin, Low-Molecular-Weight; Enoxaparin.

INTRODUCTION

Deep venous thrombosis occurs in 50% - 70% of patients submitted to acute fixation of proximal femur fractures, in multiple fractured patients, and in those presenting with spinal cord trauma, when no prophylactic measure is performed⁽¹⁾. Thrombosis may occur in any vessel of the body, but it is often found in lower limbs. This is the most important kind of thrombosis, both in terms of frequency and severity. There are some factors that may increase the risk of deep venous thrombosis, such as: age above 40 years old, extended rest periods, extensive surgeries, surgical complications, general anesthesia, immobility, trauma etc.⁽²⁾. Therefore, prophylactic measures are performed in patients at a higher risk of deep venous thrombosis, if submitted to those conditions.

The most commonly studied and used drug in prophylaxis of thrombosis is heparin⁽³⁾. Depending on its molecular weight, it is found as non-fractionated heparin or as low-molecular-weight heparin (LMWH). When compared to placebo, both the non-fractionated heparin and LMWH decrease the incidence of deep venous thrombosis in 45% of the patients submitted to hip fractures reduction and osteosynthesis⁽⁴⁾. LMWH is produced from fragments of standard heparin, by enzymatic and chemical depolymerization processes, which reduce its molecular weight by about one third. It differs from non-fractionated heparin for presenting higher levels of anti-Xa activity, higher bioavailability in lower dosages, longer half-life, increased predictability to anticoagulant response when administered in fixed dosages and for not

Study conducted by the Department of Orthopaedics and Traumatology, Cajuru University Hospital, Catholic University of Paraná

Correspondences to: Rua Canadá, 1200, Bacacheri, Curitiba, PR. CEP: 82510-290 - e-mail: salimmussi@hotmail.com

1. Assistant doctor, Orthopaedics and Traumatology Service
2. Resident doctor, Orthopaedics and Traumatology Service
3. Medicine student, 5th grade
4. Chairman

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requiring laboratory control ⁽⁵⁾. Many studies have shown that LMWH is significantly superior to non-fractionated heparin and to warfarin in preventing deep venous thrombosis and pulmonary embolism ⁽⁴⁻⁷⁾. Complications due to bleeding are significantly lower when compared to non-fractionated heparin and even lower if compared to warfarin.

However, some experimental and clinical evidences suggest that heparin interferes in bone metabolism. It is known that its extensive use may lead to a reduction of the bone mass in various amounts, and can evolve even to osteoporosis and pathological fractures ⁽⁸⁾. Its cause is still unknown. Some authors suggest that heparin may inhibit osteoblasts activity and trigger osteoclasts activities, which would lead to a progressive bone mass loss. There are some theories about the non-organization of the existent clot on fracture's core, or the inhibition of the cells responsible for bone union caused by the use of heparin, leading to union delay and increasing the risk of pseudoarthrosis ⁽⁹⁾.

There are few studies reporting the effects of LMWH on bone repair after fractures. With the purpose of evaluating the effects of this drug on bony callus formation, an experimental study was conducted on rats.

MATERIALS AND METHODS

Twenty two four-month-old male Wistar rats, weights ranging from 250 to 350 grams, were used. The animals remained in cages with two animals each, with standardized meals and water ad libitum. They were maintained under light control (bright-dark cycle of 12 hours), in a temperature of $25 \pm 1^\circ\text{C}$, and stable humidity and noise levels conditions.

This study was approved by the Committee on Ethics in Animal Research of the Catholic University of Paraná (CEPA-CCBS-PUCPR) under protocol number 022/03.

All animals were submitted to diaphyseal fracture on right femur. For this, they were anesthetized by administering, via intraperitoneal,

a solution composed of ketamin at a dosage of 40 mg/Kg and xylazin at a dosage of 5 mg/kg of body weight diluted in 1 ml physiological saline solution ⁽¹⁰⁾. Five minutes prior to surgical procedure, during anesthetic induction, the drug or saline solution, or enoxaparin started to be administered, which were repeated in a daily basis until the moment of animals' euthanasia. Animals in group I received 0.5 ml saline solution subcutaneously on their backs. Animals in group II received LMWH (sodium enoxaparin) at a dosage of 1 mg/Kg via subcutaneous on their backs ⁽⁴⁾.

Once anesthetized, the animals were positioned at dorsal decubitus in a surgical table, with extended posterior limb. The anterior trichotomy was performed on the knee and the anterior trichotomy on the right hip, with asepsis of all right posterior limb, sterile surgical drapes placement, and the following procedures were begun (Figure 1): approximately 2-cm anterior longitudinal incision on right knees; dissection by planes up to patella anterior surface; incision on the medial retinaculum of the patella and lateral dislocation; with an introducer (abocath 16 G), an initial bone perforation was performed on intercondylar space; introduction, using a battery-driven drill, of a Kirschner wire with 1.0 mm diameter, longitudinally from femur up to major trochanter; perforation of the major trochanter with that Kirschner wire and exposure at hip region, where it was moved from distal to proximal until its total introduction into distal femur; from hip region, that Kirschner wire was folded, cut and subcutaneously introduced; patellar reduction. Skin suture with nylon 4-0.

After surgical procedures, and still under anesthesia, the rats were submitted to diaphyseal fracture of right femur. In order to standardize the kind and strength required to cause fractures, a device called Fracturer was used ⁽¹¹⁾. It works as a blunt guillotine and consists of a body, a base for the animal, a system to release the bar, and a steel blunt bar of 500 g (Figure 2). The body of the device consists of a base and two platforms mounted on vertical bars. Both platforms have slots through where the steel bar goes down, which, in turn,

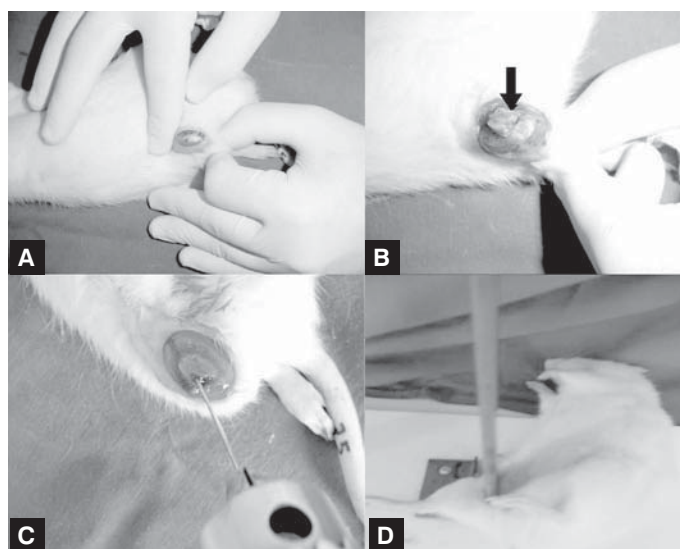


Figure 1 – Major phases of surgical procedure. A) Anterior longitudinal incision on knee, with view to patellar anterior plane. B) Patella laterally dislocated and femoral condyle view (arrow). C) Kirschner's wire (with a longitudinal diameter of 1.0 mm) passing through the femur. D) Experimental animal positioned with right posterior limb abducted on metal bases, being submitted to femur fracture.

is released from a 30-cm height by releasing a lock. Its fall is stopped by another lock; this one limits the fall at 1 mm beyond bar's contact point to animal's femur, thus restricting angular deformation of both the bone and the intramedullary wire. The animal is positioned in dorsal decubitus, with abducted posterior limb and with the region to be fractured (femoral diaphysis) positioned over two metal bases. During bar fall, it progresses at the middle of the platform slots, which form its support point, to keep on its track. Its path

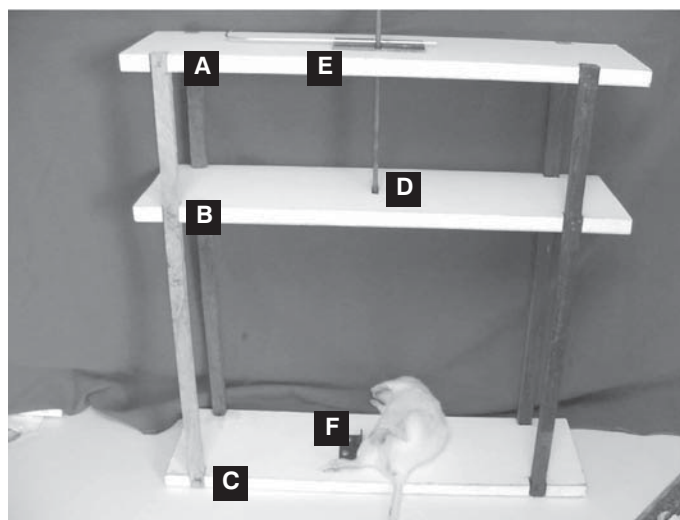


Figure 2 – Picture of the Fracturer device. It is built with three wooden plates (A, B and C) measuring 12.5 x 45 cm each, 30-cm height (from B to C) up to bar detachment site (D), weighting 500 g and has a lock in its upper portion, limiting fall. A second platform at 10 cm (a) to the first one stabilizes the assembly and the impacting bar contains a device (E) releasing the bar. Animal's femur rests on two metal bases (F – according to figure 3).

ends at the middle of the rat's femoral diameter and at the center of the two metal bases, forming a three-point system, producing a closed and standardized fracture.

Rats were also submitted to X-ray studies on their right femurs, which was performed with the aid of a portable device, with ampoule positioned at 80 cm from the frame, with a 48-mA load 25 watts, to determine the place and configuration of the fracture (Figure 3). Full support on fractured limb was allowed during postoperative period.

After four weeks, they were submitted to euthanasia with intraperitoneal anesthesia with barbiturate, finishing with a lethal dose of potassium chloride.

The study of femoral bone union was performed by means of macroscopic and histological evaluations ⁽¹²⁾.

Macroscopic evaluation was performed based on parameters for assessing femoral diaphysis union. Macroscopic union was established as the presence of bony callus at fracture core and the union of femoral fragments, and the non-union was established as an absence of bony callus and the non-union of femur fragments.

At bony callus histological evaluation, fibrous tissue, cartilaginous tissue, and bone tissue formation were

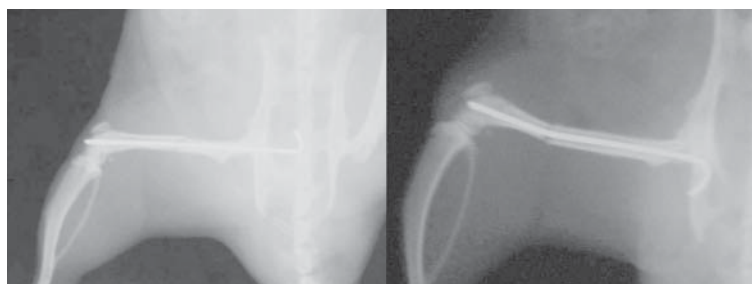


Figure 3 – Postoperative X-ray images of fractured femurs.

examined. All slides were assessed by the same pathologist, who was blind to which group the material under analysis belonged to (study or control). Every slide was visualized and then, the percentage for each tissue was examined. As an evaluation parameter, a comparative study between groups I and II was performed.

For comparing groups regarding union or non-union evidences (macroscopic analysis), the Fisher's exact test was used. For comparing macroscopic analysis to the

percentage of fibrous, cartilaginous and bony tissue, the Mann-Whitney's non-parametric test was used. P values < 0.05 were considered as statistically significant.

RESULTS

In the 22 animals studied, we found no serious complications related to surgical procedure, or during the 28-day postoperative period up to the moment of euthanasia.

After the 12th postoperative day, in average, animals presented with a good mobility on right posterior limb and load could be applied on it during ambulation.

Table 1 shows macroscopic evaluation data regarding femoral fractures union or non-union. There was no statistical difference between both groups.

At histological evaluation, bony callus formation was seen in all 22 femurs assessed. A femur that was macroscopically classified as in non-union also showed bony callus formation microscopically. The femoral diaphysis presented with normal

and organized bony trabeculate, and, as that trabeculate was coming closer to fracture core, it was no longer much organized, also presenting, at the central region, cartilaginous and fibrous tissues in the majority of cases (Figures 4 and 5).

On Table 2, descriptive results and p values are shown comparatively to the histological quantification of the numeric percentage for each kind of tissue: bony, cartilaginous, and fibrous. By analyzing those data, we see that there was no statistical difference between control and study

groups. In other words, at histological evaluation, bony callus formation was similar for both groups.

Infection foci were found in four femurs, being two from group I and two from group II. But, microscopically, no changes were found on bony callus formation. That was present in all cases.

DISCUSSION

Anticoagulants must be routinely used in patients presenting risk factors to deep venous thrombosis, as well as in those experiencing fractures at pelvic region, at lower limbs and in cases of multiple traumas. Anticoagulant drugs have been quickly recycled, and there is no universally accepted and adopted protocol yet. LMWH and warfarin, at low dosages, are being used for replacing the traditional non-fractionated heparin. Both LMWH and warfarin represent the most efficient drugs to fight deep venous thrombosis, with the LMWH advantage of not requiring laboratory control for dosage adjustment (6,13).

The use of LMWH for thrombosis prophylaxis reduces the risk of deep venous thrombosis by 45% to 66% and reduces the risk of death due to pulmonary thromboembolism in up to 50% (4,6). However, it is crucial that thrombosis prophylaxis starts early, within up to 24 hours after trauma. The delay in starting thrombosis prophylaxis significantly increases the risk of deep venous thrombosis (7).

Osteoporosis is an undesirable con-

Groups	Fracture				Total	
	Union		Non-Union			
	N	%	N	%	N	%
Group I (control)	11	100.0	0	0.0	11	100.0
Group II (study)	10	90.9	1	9.1	11	100.0
Total	21	95.5	1	4.5	22	100.0

Fisher's test $p = 1$

Table 1 – Macroscopic correlation between united and non-united femoral fractures.

of pseudoarthrosis in four patients receiving anticoagulant therapy for thrombophlebitis immediately following surgery. Therefore, they conducted the first experimental study in an attempt to determine the potential cause relationship between anticoagulants and bone union failure. In practice, comparative clinical evaluation of bony callus formation in fractures brings a lot of difficulties, because

there are individual differences concerning the nature and site of injury, course and duration of union. And it is also especially difficult to study therapeutic methods able to influence bony callus formation. Thus, it is clear why this subject is experimentally addressed, for us to be able to homogeneously compare the studied materials, that is, the same conditions regarding gender, age, weight, nutrition, nature and site, kind and mechanism of fracture.

The drug used in this experimental study intended for analyzing its potential influence on fractures union is enoxaparin, which is a low-molecular-weight heparin. Dosage used was similar to a prophylactic dosage used for an adult human being (1mg/kg/day) (4). Dosage was adjusted according to each rat's weight. All rats were weighted prior to surgical procedure and at a weekly basis, thereafter.

Anesthesia performed via peritoneal is the most commonly used for rats (10). During anesthesia in our experiment, no complications or difficulties occurred. Therefore, this way was

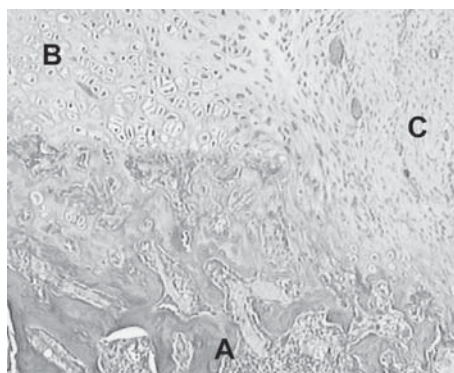


Figure 4 – Photograph showing histological aspects of bony callus in a fractured femur after four weeks, in a control group rat. Stain: HE. Magnification: 100 x. A) Bone tissue. B) Cartilaginous tissue. C) Fibrous tissue.

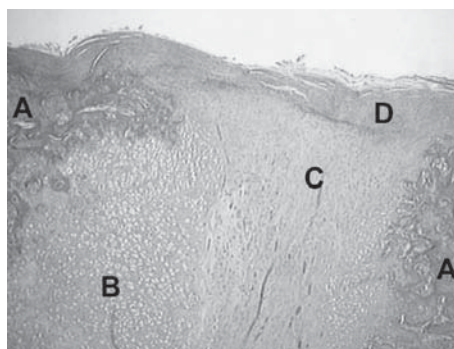


Figure 5 - Photograph showing histological aspects of bony callus in a fractured femur after four weeks in a study group rat (with heparin). Stain: HE. Magnification 50 x. A) Bone tissue. B) Cartilaginous tissue. C) Fibrous tissue. D) Periosteum.

proven to be efficient and can be used for this kind of animal.

In order to standardize the kinds of fractures and to avoid that its performance could interfere in our research outcomes, we used the Fracturer, which was developed by Vialle et al.⁽¹¹⁾. With that device,

we were able to fracture femoral diaphysis in rats always applying the same injury-causing force. The comparison on fractures union requires similarity, which was pursued in this study. Animals were not immobilized, but remained free for ambulation, as much as the lower limb would allow, because immobilization was considered unnecessary.

According to Udupa and Prasad⁽¹⁴⁾, experimental fractures on rats' femurs reach the ossification phase approximately at the fourth week, when osteogenic evidences of union could already been seen. Those authors defined four phases of fractures union process in rats: first week, fibroblastic phase; second week, collagen phase; third and fourth weeks, osteogenic phase, and; fifth and sixth weeks, remodeling phase. Based on these data, euthanasia was determined to the 28th day after fractures, because, during the osteogenic phase, true union happens, and our objective was to evaluate whether enoxaparin influenced bone union or not.

A single similar study was found in literature indexed to Medline, in which the effects of LMWH administered for thrombosis prophylaxis on bone union was one by

Variant	Group	Minimum %	Maximum %	Average %	P value %
Fibrous	Control	5	45	20.0	0.2703
	Study	0	60	17.3	
Cartilaginous	Control	5	20	10.5	0.8470
	Study	0	35	12.3	
Bone	Control	45	80	69.5	0.8470
	Study	40	90	70.5	

Table 2 – Correlation of fibrous, cartilaginous and bone tissue, according to histological criteria.

Street et al.⁽¹⁵⁾. Closed fractures were manually performed in rabbits' ribs. They concluded that bone repair was significantly attenuated in all animals receiving subcutaneous enoxaparin when compared to control animals. However, it differs for the kind of animal, fractured

bone, fracture control, and, mostly, for the bony callus evaluation period. They performed histological evaluation up to the second week after fracture. According to Udupa and Prasad⁽¹⁴⁾, within that period, bony callus is still in the collagen phase. We preferred to study bone repair formation during the osteogenic phase, around the fourth week, because it is during that period that we can determine if the fracture is or is not united.

Evident suggestions were found in literature regarding LMWH's potential to delay bone metabolism, which may, in more severe degrees, evolve to osteoporosis or even to pathological fractures.

LMWH use effects in bone union process are still little studied. Few studies state that it acts by reducing bone repair. In this experiment, we could not corroborate such statement, because it did not interfere on bone union process.

CONCLUSION

Low-molecular-weight heparin (enoxaparin) did not influence bony callus formation process in fractures on rats' femurs.

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