The Effect of Low Molecular Weight Heparins on Fracture Healing

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Abstract: Venous Thromboembolism is a serious complication in the trauma patient. The most commonly studied and used anticoagulant treatment in prophylaxis of thrombosis is heparin. The prolonged use of unfractionated heparin has been connected with increased incidence of osteoporotic fractures. Low molecular-weight-heparins (LMWHs) have been the golden rule in antithrombotic therapy during the previous two decades as a way to overcome the major drawbacks of unfractioned heparin. However there are few studies reporting the effects of LMWHs on bone repair after fractures. This review presents the studies about the effects of LMWHs on bone biology (bone cells and bone metabolism) and underlying the mechanisms by which LMWHs may impair fracture healing process. The authors' research based on literature concluded that there are no facts and statistics for the role of LMWHs on fracture healing process in humans and the main body of evidence of their role comes from *in vitro* and animal studies. Further large clinical studies designed to compare different types of LMWHs, in different dosages and in different patient or animal models are needed for exploring the effects of LMWHs on fracture healing process.

Keywords: Fracture healing, fractures, heparin, low molecular weight heparin.

INTRODUCTION

The most common cause of death and morbidity in the trauma patient are the thromboembolic complications [1, 2]. 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. The most studied and used drug in prophylaxis of thrombosis is heparin [3]. Heparin- induced osteoporosis after long- term and high- dose usage of unfractioned heparin (UFH) has been considerably investigated too [4-6].

Low molecular-weight-heparin (LMWH) was developed in the decade of 80s to overcome some of the major disadvantages of unfractioned heparin. During these years, a new question has been raised as to whether LMWHs carries the same side effects compared to standard UFH [7]. Unfractioned heparin and LMWHs have been shown to have several harmful effects on bone, causing osteoporosis and enhancing the bone resorption. Moreover, both of them seems to increase calcium loss and reduce bone turnover [8-11]. However, there are few studies reporting the effects of LMWH on bone repair after fractures. Due to the fact that a great number of trauma patients with fractures regularly receive LMWH, it is essential to examine whether LMWH may have an adverse impact on fracture healing process.

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LMWHs: HISTORICAL PERSPECTIVE AND OVERVIEW

Heparin, a highly sulfated glycosaminoglycan, was discovered to have antithrombotic properties by Mc Lean nearly 100 years before [12, 13]. Brinkhous *et al.* [14] then proved that heparin is an indirect anticoagulant, requiring a plasma cofactor- antithrombin (AT) III or simply referred as AT. The heparin/AT interaction causes a conformational change in AT. The activated AT then inactivates the coagulation enzyme thrombin (factor IIa) and other proteases involves in blood clotting most notably factor Xa and factor IXa. The molecular weight of heparin varies from 5000 to 30000 with a mean molecular weight of 15000 (approximately 50 monosacharide chains) [12].

LMWHs are polysulfated glycosaminoglycans which are almost one third the molecular weight of UFH and they are derived from UFH by chemical or enzymatic polymerization. LMWHs have an average molecular weight of 4000 to 5000 (about 15 monosacharide units per molecule). Due to the fact that LMWHs are prepared by different methods of depolymerization of heparin, they differ in many factors such as pharmacokinetic properties, anticoagulant profiles and they are not clinically interchangeable [13]. Thus, LMWHs are a group of similar but different drug agents. The various LMWHs approved for use are shown in (Table **1**). It is estimated in a large number of studies that the use of LMWHs is the best way to prevent dangerous clinical complications like venous thrombosis or acute pulmonary embolism [13]. Especially in pregnancy the use of LMWHs is considered the golden rule in anticoagulant therapy. Also LMWHs are used in obstetrics for the prevention of first

trimester loss or the placental dysfunction in women suffering from thrombophilia [15]. Finally, other advantages of LMWHs over standard heparin are the longer plasma half life and the fact that they have more predictable anticoagulant response. Also, it is of great importance that they require less intense laboratory monitoring [16].

Table 1. Different types of LMWH.

LMWH	Average Molecular Weight in Daltons
Ardeparin	5500-6500
Bemiparin	3600
Certoparin	5400
Dalteparin	6000
Enoxaparin	4500
Nadroparin	4300
Parnaparin	5000
Reviparin	4400
Tanziparin (Logiparin)	6500

FRACTURE HEALING PROCESS

Fracture healing is a complicated process that involves the coordination of a sequence of many biological events [17]. This process requires the action of appropriate cells such as osteoblasts, osteoclasts, fibroblasts, chondroblasts, macrophages, monocytes and lymphocytes. Moreover, a number of genes play an important role to this process including growth factors, transcription factors and genes that control matrix production and organization [18]. In 1975, Cruess and Dumont [19] suggested that fracture healing consisted of three phases the one following the other: an inflammatory phase, a reparative phase and a remodeling phase. In 1989, Frost [20, 21] suggested five stages of fracture healing: stage of hematoma, stage of granulation tissue, stage of callus formation, stage of modeling and stage of remodeling. For convenience, fracture healing will be described as recognized by Cruess and Dumont stressing the fact that the reparative phase is a combination of many processes (the stage of granulation tissue and the stage of callus formation) (Table **2**). Moreover, although we can divide the fracture healing process into phases, we must draw attention to the fact that what we describe happened in one phase is noticed in the following one as well, and that events seen in phases that follow, were marked in a previous phase.

LMWHs AND BONE BIOLOGY

The high dose and long term use of heparin has been recognized by scientists as a risk factor for the development of osteoporosis and osteoporotic human fractures [22-24]. Data on osteoporosis associated with LMWHs are contradictory [25, 26]. Most of the reported cases of symptomatic osteoporosis with spontaneous fractures occurred in pregnant women treated with UFH recurrent thromboembolism [27]. Dalhman *et al.* [4] reported that a rate 2,2% of patients receiving UFH had osteoporotic spinal fractures. Several factors can cause heparin induced osteoporosis. Some of the most important are the variations in the metabolism of vitamin D and the high rate of bone resorption. Another factor is the decreased activity of osteoblast either the overactivation of osteclasts [28, 29].

Osteblasts and osteclasts are responsible for bone homeostasis (bone formation and resorption) respectively. The use of UFH may disturbs the maintain balance between these two major cell types and causes heparin- induced osteoporosis.

Moreover, osteoblasts number and function is very important for the integration of endoprosthetic implants, the permanent remodeling processes of bone but also for the process of fracture healing. Recent studies prove that osteoblasts arise from mesenchymal stem cells [30]. Mesenchymal stem cells (MSCs) are extremely proliferative stromal cells that have the ability of forming bone and cartilage. They play an important role in bone homeostasis because they are an appropriate source for osteoblasts [31, 32]. Furthermore, except from osteoblasts, mesenchymal stem cells may differentiate *in vitro* or *in vivo*, into variety of cell type's inclunding: chondryoblasts, adipocytes and muscle cells [30]. Which factors are responsible for regulating the differentiation of mesenchymal stem cells are to large extent under investigation. However, current studies suggest that a number of cytokines are responsible for the physiological differentiation of (MSCs) into mature osteblasts including interleukin (IL), IL 1, IL-6, IL-11 and tumor necrosis factor (TNF) α [30]. It is possible therefore, that heparin alters the expression of one or more of these cytokines [33]. By the same mechanism maybe LMWHs impair bone metabolism.

Table 2. Phases of fracture healing.

	Stages of Fracture Healing	Cells and Genes Involved	% of the Total Healing Time of a Fracture
Inflammatory Phase	- Hematoma - Intense inflammation	Lymmphocytes, platelets, blood monocytes, macrophages, osteoclasts. TGF-β, FGF-I, FGF- II, PDGF, osteonectin, IGF-I, IGF-II, IL-1, IL-6.	10%
Reparative Phase	- Granulation tissue - Fibrocartilage callus formation - Bony callus formation	Macrophages, osteoblasts, osteoclasts, chondroblasts, chondrocytes, fibroblasts. TGF-β, FGF-I, FGF- II, PDGF, IGF-I, IGF-II, osteonectin, osteocalcin, IL-1, IL-6, collagens (different types).	40%
Remodeling Phase	Morphological adaptation of bone to regain optimal architecture, function and strength		70%

In addition, osteoblast proliferation in humans is affected by many growth factors, insulin-like growth factors (IGFs) I and II included [34]. In human osteoblasts we can find a surface binding protein for IGFs (IGF binding protein 5) to which heparin could bind [35]. Heparin binding happens competitively with the binding of other IGFs and that might explain the impaired regulation of osteoblast differentiation and possible changes in the formation of bones after long-term treatment with heparin [36].

Finally, osteoclasts play an important role on bone biology. Osteoclasts are multinucleated cells that resorb bone tissue and they are developed from macrophages. They are regulated by several factors including 1, 25 dihydroxyvitamin D3, parathormone (PTH), interleukin 11 (IL-11). Also osteoclasts formation requires the presence of receptor activator of nuclear factor kB (RANKL) and macrophage colony stimulating factor (M-CSF) [37-39].

This review presents the studies about the effects of the LMWHs on bone biology (bone cells and bone metabolism) and highlights the underlying mechanisms by which LMWHs may impair fracture healing (Fig. **1a**, **b**).

LITERATURE REVIEW

Methodology

We carried out a systematic review of the effect of LMWHs on fracture healing process by searching the electronic data bases PubMed, Google search and Google Scholar, Heal Link, EMBASE, Scopus, Cochrane Library, up to July 2013. The search terms were: 'Low- molecular weight heparin', 'Low molecular weight heparins', 'LMWH', 'LMWHs', 'fracture healing', 'fracture healing process', 'Bemiparin', 'Certoparin', 'Dalteparin', 'Enoxaparin', 'Nadroparin', 'Parnaparin', 'Reviparin', 'Tanziparin', 'LMWHs side effects', 'Low- molecular weight heparins osteoporosis', 'Low- molecular weight heparins fracture healing', 'Low- molecular weight heparins osteoblasts', 'Low- molecular weight heparins osteoclasts', 'effects of Low- molecular weight heparins on fracture healing'. All the articles have been evaluated and supplemented by searches of the bibliographies of key papers.

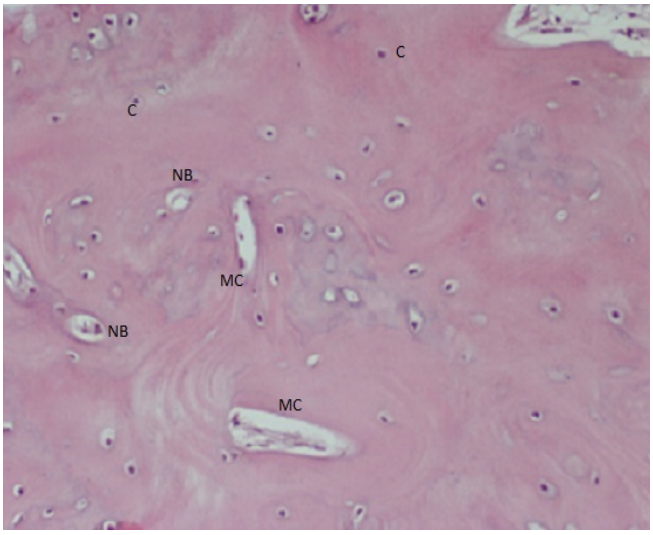
Results

A total of 28 articles were identified investigating the effects of LMWHs on fracture healing process through electronic database searches. However, only 8 studies concern *in vivo* animal model, the rest 20 concern bone biology (bone cells and bone metabolism) and they are presented to elucidate possible mechanism by which LMWHs impair fracture healing.

Studies Concerned LMWHs and Fracture Healing Process In Vivo Animal Models

The findings of *in vivo* animal models studies with regard to the effect of LMWHs into fracture healing remain conflicting (Table **3**). Stinchfield *et al.* [40] first reported the effects of anticoagulant therapy on bone repair. They showed that administration of heparin or warfarin on a daily basis attenuated bone repair in rabbits and canines significantly. Using histologic analysis, they saw an increase in fibrous tissue and absence of bony bridging in the callus of animals treated with either heparin or warfarin.

(**a**)



(**b**)

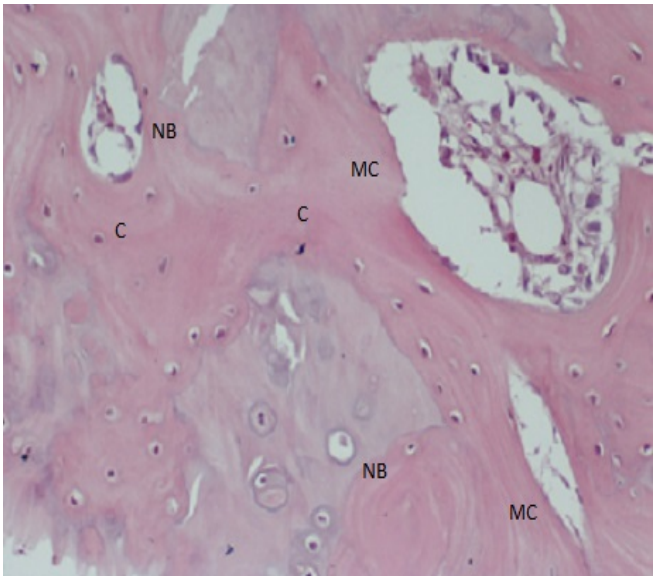


Fig. (1a, **b).** Bone formation (callus) after Low Molecular heparin therapy in rabbits. Undecalcified histological sections (**a**, **b**) of a critical-sized defect treated (**a**) with heparin (**b**) without heparin 2 months after the fracture. (HxE, magnification X100) NB: new bone, MC: medullary cavities, C: cartilage.

On the other hand, the effects of LMWHs on fracture healing were first suggested by Street *et al.* in 2000 [41]. They studied the effects of one LMWH (enoxaparin) on the fracture healing process in a closed rabbit rib fracture and they mentioned a significant delay. More specific, they evaluated the fracture healing using different methods such as histomorphometric, histologic and immune histological included. Moreover, biomechanical testing with torsional loading was assessed after 21 days. Fracture healing was significantly attenuated in every case in rabbits which received subcutaneous enoxaparin when compared with that of the control group. The authors of this study made the

Table 3. LMWHs- effects on fracture healing process.

Authors	Journal/Year	LMWH type	Animal Model	Dose	Results
Street J [41]	Clinical Orthopaedics (2000)	Enoxaparin	Rabbits	2 mg (in 400 μL normal saline)	Bone repair was notably attenuated in animals subcutaneous enoxaparin compared with the control group
Kock HJ [42]	Unfallchirurg (2002)	Certoparin	Rabbits	40 IU/kg	The influence of heparins on fracture healing process can be reduced remarkably by using LMWH instead of UFH
Curcelli EM [64]	Acta Orth Bras (2005)	Enoxaparin	Rats	1 mg/kg	Histological and biomechanical evaluations showed that the administration of enoxaparin and heparin sodium did not intervene in bone consolidation in rats
Erli H [43]	Journal of Orthopedic Surgery (2006)	Dalteparin Certoparin	Rabbits	50 anti Xa units/Kg/day	Dalteparin and Certoparin caused a non-specific reduction in bone healing rate compared to the control group
Hak D [44]	Journal of Orthopaedic Research (2006)	Dalteparin	Rats	70 Units/Kgr	Dalteparin did not impair fracture healing process in rats femure
Filho S [45]	Acta Orth Bras (2006)	Enoxaparin	Rats	1 mg/kg	LMWH (enoxaparin) did not influence bony callus formation process in fractures on rats femurs
Demirtas A [46]	Eur Rev Med Pharmacol (2013)	Enoxaparin	Rats	1000 anti Xa IU/kg	Enoxaparin, fondaparinux and rivaroxaban used in thrombo embolism prophylaxis cause no significant changes in fracture healing
Say F [47]	Thromb Rest (2013)	Enoxaparin Nadroparin Dalteparin	Rats	Enoxaparin 1mg/kg Nadroparin 200 u/kg Dalteparin 140 u/kg	An assertive histological effect of fondaparinux on fracture healing process was noticed

hypothesis that thromboprophylaxis using enoxaparin would delay fracture healing by interfering with two distinct processes of bone formation. First, by binding to the vascular endothelium, enoxaparin would disrupt callus vascular disassembly and the transformation of pericytes to osteoprogenitor units. Second by virtue of an increased bleeding tendency, LMWH would promote intefragmentary hematoma collection, increasing cytoxicity to cells in the medullary callus, thus delaying bone formation during fracture healing.

Because Street *et al.* used enoxaparin, Kock *et al.* [42] used certoparin in a blinded trial. They caused bone defects to both femur condyles of rabbits and they divided them into three groups. The first group received subcutaneous injections of sodium heparin, the second group received enoxaparin and the third one, normal saline for a period of six weeks. After this period the defects at group treated with UFH remained significantly larger in depth compared to group treated with LMWH, in which there was no inhibition of defect healing. The result of this study showed that the use of LMWH instead of UFH is preferable because reduces significantly the negative influence of heparins on fracture healing process.

On the other side, Erli *et al.* [43] investigated the effects of two LMWHs (dalteparin and certoparin) on fracture healing process. Female rabbits defined metaphyseal defects to their femora. Then they were injected with either saline solution, unfractioned heparin or one of two different LMWHs for a six weeks period. In this study at clinical relevant doses of LMWH it was proved no- specific reduction of bone healing.

Similar to Street *et al.*, Hak *et al.* [44] studied the effects of dalteparin on fracture healing process. This time used a stabilized rat femur fracture model. They assessed the fracture healing process by radiographs, histology and mechanical testing. It was the second study after Street *et al.* that evaluate the mechanical properties (maximum torque, stiffness and energy absorption to maximum torque) in facture healing. They concluded that in the LMWH group the mean maximum torque and mean stiffness, approach that of the intact femurs after six weeks. Unlike to the findings of Street *et al.* [41], dalteparin at the dosage used in this study, did not impair the fracture healing process and did not have any effect on fracture healing mechanical properties.

A study by Filho *et al.* [45] investigated the effects of LMWH (enoxaparin) on the formation of bony callus in rats' femurs. Wistar male rats were submitted to diaphyseal fracture on the right femurs. One group of rats received saline solution while the study group received enoxaparin daily during the time of 28 days. At histological evaluation, bony callus formation was similar for both groups. It was

concluded that enoxaparin does not cause any changes on the fracture healing process.

Demirtas *et al.* [46] investigated the effects of enoxaparin, fondaparinux and rivaroxaban on fracture healing in a rat model of femur fracture. In this study, enoxaparin caused no significant changes in fracture healing process.

Finally, Say *et al.* [47] investigated the effect of enoxaparin, nadroparin, dalteparin and fondaparinux on fracture healing. In this study it was observed only an enhancing histological effect of fondaparinux on fracture healing process because of non- inhibitory effect on osteoblasts and growth factors.

Studies Concerned LMWHs and Bone Biology

Despite the limited number of *in vivo* animal studies about the effect of LMWHs on fracture healing process, there is a large number of *in vitro* studies on bone cells and bone metabolism demonstrate that LMWHs decrease bone formation and therefore, could potentially delay fracture healing (Table **4**).

Studies Concerned LMWHs and Bone Cells

The main cells for bone formation are osteoblasts. Kock *et al.* [7] reported a significantly inhibitory effect of different LMWHs on human osteoblast growth *in vitro*. In the same way, Osip *et al.* [31] demonstrated that LMWH inhibit osteoblast formation and promote adipocytes differentiation but to a lesser extent than heparin because these activities were found to be both chain- length and charge- dependent.

In previous study, Muir JM *et al.* [48] suggested that both heparin and tinzaperin had the tendency to decrease bone formation by decreasing osteoblast number and activity, but that only heparin increases osteoclast differentiation and activity.

Handschin *et al.* [36] noticed that when human osteoblasts cell culture incubated with dalteparin, the osteoblast proliferation was inhibited. They also mentioned that two other factors osteocalcin and alkaline phosphatase (ALP) were inhibited too. These two regulators are crucial in maintaining the bone homeostasis. Human osteoblasts have a high amount of ALP anchored in their outer surface. ALP is a biochemical marker of osteoblast activity and regulates osteoblast differentiation. Therefore, ALP levels reflect the rate of bone formation. On the other hand, osteocalcin is a protein that regulates osteoblast differentiation and maturation. The authors come to the conclusion that high doses of dalteparin causes the inhibition of these two major regulators and in their turn causes inhibition of osteoblast differentiation, leading finally to heparin induced-osteoporosis.

In another study, Bhandari M *et al.* [49] examined the effects of heparin and LMWH (enoxaparin) on osteoblasts function and the ALP activity. LMWH and heparin inhibited osteoblast function (bone formation) but LMWH required in higher concentrations to achieve equivalent effect. Enoxaparin produces less inhibition of bone nodule formation than heparin because this activity is both chain-length and charge dependent.

Matziolis *et al.* [50] examined the effects of fondaparinux on osteoblasts. UFH, dalteparin, enoxaparin and fondaparinux were added to osteoblast cultures. The use of fondaparinux showed a significant affection at protein synthesis and mitochondrial activity of osteoblasts. Unlike dalteparin, enoxaparin, and UFH lead to significant decrease of matrix collagen type II content and calcification.

Except from osteoblasts, osteoclasts play a significant role on fracture healing process. Chodhurry *et al.* [37] first demonstrated the fact that low doses of standard heparin directly stimulated bone resorption by increasing the number and the activity of osteoclasts.

In the same way, Walton *et al.* [38] concluded that heparin has a synergistic effect with cytokine interleukin 11 (IL-11), which leads to increased osteoclast formation and activity.

Moreover, Folwarzna *et al.* [39] showed that the effects of standard heparin and all investigated LMWHs (Nadroparin, Enoxaparin, Dalteparin, Parnaparin) on osteoclast formation follow similar patterns. All heparins (standard and LMWHs) was proved to influence the formation of osteoclasts in two directions. At lower concentrations tended to increase the osteoclast formation, whereas at the highest concentrations they tended to decrease or did not affect the osteoclast formation.

Studies Concerned LMWHs and Bone Metabolism

There is a consensus from a number of studies that both heparin and LMWH affect bone metabolism especially bone density and weaken the biomechanical properties of bone (Table **5**).

Nishiyama *et al.* [51] studied the effects of heparin and dalteparin on bone metabolism in rats. They injected intravenous heparin and dalteparin in rats for 28 days. After this period in the heparin treated group observed significant loss of bone weight and mineral contents (calcium, phosphorous). On the other side, the rats treated with dalteparin slightly reduced bone mass. They also observed that in the heparin treated group 7 out of 8 rats had fractures on femora while at the dalteparin group no rat femur had broken. In conclusion, the study shown that dalteparin produce a weaker effect on bone resorption and formation compared with heparin.

Shaugnessy G *et al.* [52] adapted a reproducible experimental model to quantify heparin- induced calcium loss from bone. They determined that both size and degree of sulfation were the major factors of heparin's ability to affect bone resorption. Heparin seems to stimulate collagen synthesis in osteoblast cultures [53]. Moreover, heparin has a synergistic effect with PTH, stimulating bone resorption in organ cultures and interacts with unknown serum factors to stimulate bone resorption by disaggregated osteoclasts. They found that LMWHs produced significantly less calcium loss than UFH and proposed that the use of LMWHs instead of UFH reduce the risk of heparin- induced osteoporosis.

Murray *et al.* [54] observed a reduction in trabecular and cortical bone of rabbits at treatment with UFH and HMWH but not with LMWH. The use of HMWH also increased significant the percentage of femur fractures in rabbits. They

Table 4. Characteristics of all articles with LMWHs- effects on bone biology.

Authors	Journal/Year	LMWH Type	Animal Model	Dose	Effect	Results
Monreal M [6]	Haemostasis (1990)	Dalteparin	Rats	1 anti Xa U/g	Bone metabolism-density	LMWH may produce less osteopenia than that of standard heparin
Murray WJ [54]	Blood Coagul Fibrinolysis (1995)	CY 216 Choay Laboratories (Fraxiparin)	Rabbits	750 anti Xa U/Kg	Bone metabolism-density	In contrast to UFH or HMWH, the prolonged administration of LMWH in high daily dosages does not cause osteoporosis in rabbits.
Shaugnessy S [52]	Blood (1995)	Enoxaparin Dalteparin Tanziparin Ardeparin	Rats	14.0 anti Xa units/ml	Bone metabolism-density	The LMWHs may cause remarkable less calcium loss than classic heparin
Muir J [48]	Blood (1997)	Tinzaparin	Rats	1.0 U/g or 0.5 U/g	Bone metabolism-density	Heparin and Tinzaparin decrease osteoblast and osteoid surface (bone formation) to the same extent but only heparin increases osteoclast surface (bone resorption)
Nishiyama M [51]	Jpn. J. Pharmacol. (1997)	Dalteparin	Rats	anti- factor Xa 1000, 3000 and 10000 U/2ml/Kg	Bone metabolism-density	Dalteparin compared to heparin produced a weaker effect on bone resorption and formation
Bhandari M [49]	Thromb Haemost (1998)	Enoxaparin	Rats	100 U/mg	Bone metabolism-density	LMWH and heparin inhibited osteoblast function (bone formation) but LMWH required in higher concentrations to achieve equivalent effect
Kock HJ [7]	Clin Appl Thrombosis/ Hemostasis (2002)	Nadroparin Enoxaparin Dalteparin Certoparin	Human	Same doses 50 mg/ml	Bone cells	LMWHs caused a significant inhibition of osteoblast growth
Wawrzynska L [29]	Pathophysiol Haemost Thromb (2003)	Nadroparin Enoxaparin	Human	Nadroparin 15000IV/day Enoxaparin 1 mg/kg/day	Bone metabolism-density	Decrease in BMD observed after long term administration of nadroparin
Matziolis G [50]	Calcif Tissue Int (2003)	Dalteparin Enoxaparin	Human	0.1-1 IU/ml	Bone cells	Enoxaparin, dalteparin and UFH lead to noteworthy decrease of matrix collagen type II content and calcification in concentrations equal or higher than the therapeutic one
Osip SL [31]	Thromb Haemost (2004)	Dalteparin	Rats	100 anti-factor Xa U/ml	Bone cells	LMWH was found to inhibit osteoblast formation and to stimulate adipocyte differentiation to a lesser extent than heparin
Folwarczna J [55]	Thromb Haemost (2004)	Nadroparin Enoxaparin	Rats	1000 or 2000 anti Xa IU/Kg	Bone metabolism-Mechanical Properties	The present study indicating the unfavourable effects of LMWH on mechanical properties of bones. LMWH may differ in terms of their damaging effect on the skeletal system
Folwarczna J [65]	Pol J Pharmacol (2004)	Nadroparin	Rats	1000 or 2000 anti Xa IU/Kg	Bone metabolism-density	Nadroparin and heparin caused similar changes in the investigated bone histomorphometric parameters

(Table 4) contd…..

Authors	Journal/Year	LMWH Type	Animal Model	Dose	Effect	Results
Folwarczna J [66]	Pol J Pharmacol (2004)	Enoxaparin	Rats	1000 or 2000 anti Xa IU/Kg	Bone metabolism-density	The remarked changes in bone histomorphometric parameters suggest that enoxaparin caused the inhibition of bone formation and intensification of bone resorption
Folwarczna J [39]	Pharmacol Rep (2005)	Nadroparin Enoxaparin Dalteparin Parnaparin	Rats	1-1000 anti Xa IU/Kg	Bone cells	Standard heparin and LMWHs tended to increase the formation of osteoclasts, while at the highest concentrations they tended to decrease it
Handschin A [36]	British Journal of Medicine (2005)	Dalteparin	Human	30, 300 or 900 μg/ml	Bone cells	Dalteparin caused a remarkable dose- dependent inhibition of osteoblast proliferation
Handschin A [67]	Clin Appl Thromb Hemost (2006)	Dalteparin	Human	30, 300 or 900 μg/ml	Bone cells	Dalteparin caused a remarkable inhibition of both Cbfa-1 expression and osteocalcin *in vitro* at high dosages
Winkler T [68]	Open Orthop J (2011)	Dalteparin	Human	0.2-0.5 IU/ml	Bone cells	Melagatran affected human osteoblasts to a lesser extent, comparable or even less than dalteparin
Papathanasopoulos A [30]	Journal of Orthopaedic Research (2011)	Tinzaparin	Human	0.5 IU/ml 5 IU/ml 50 IU/ml	Bone cells	Tinzaparin treatment reduced MSC proliferation which could have implications in the initial MSC stages of fracture healing process
Sudrova M [56]	Clin Appl Thromb Hemost (2011)	Enoxaparin	Human	4000 IU/ml	Bone metabolism-density	Enoxaparin decreases the concentration of bone specific ALP
Sarahrudi K [57]	International Orthopaedics (2012)	Enoxaparin	Human	40-60 mg/ml	Bone metabolism	Remarkable difference of the expression of M-CSF and TGF- β1 after administration of enoxaparin were noticed without any influence on fracture healing process

concluded that in contrast to UFH and HMWH, the prolonged administration of LMWH in high daily dosages does not cause osteoporosis in experimental animals.

These findings were in accordance with a previous study. Monreal *et al.* [6] treated rats with UFH and dalteparin and reported that both heparins decreased bone mineral density but that the effects of dalteparin were less severe. However, in a separate study Matzsch *et al.* [9] reported that logiparin and UFH decreased bone density to a similar extent.

Muir *et al.* [48] investigated the effects of heparin and LMWH (tinzaparin) on cancellous bone in rats. They measured urinary type I collagen cross- linked pyridinoline (PYD) and serum alkaline phospatase (ALP). PYD and ALP are markers of bone resorption and formation, respectively. They come to the conclusion that heparin causes cancellous bone loss in a significantly greater extent than tinzaparin.

Folwarzna *et al.* [55] compared the effects of heparin and two LMWHs (nadroparin and enoxaparin) on bone mechanical properties in rats. They examined the mechanical properties and other parameters such as bone mass, length, diameter, mineral content in the whole femur and femoral neck of rats. They observed that the use of standard heparin weakened the femoral neck. Enoxaparin and the higher doses of standard heparin and nadroparin induced similar adverse changes in mechanical properties of whole femur.

In a recent study, Sudrova M *et al.* [56] tried to evaluate the effects of prolonged use of enoxaparin in pregnant women with thrombophilia. They measured the concentrations of bone turnover markers including osteoprotegerin (OPG), total serum alkaline phosphatase (total ALP), bone alkaline phosphatase (bone ALP), and the receptor activator of nuclear factor κB lingand (RANKL). Bone ALP is a glycoprotein found on the surface of osteblasts and reflects the bone formation activity. Osteoprotegerin is a basic glycoprotein that has a heparin binding site and is a decoy- receptor for receptor activator of nuclear factor κB lingand (RANKL). RANKL plays a critical role for activation, development and maturation of osteoclasts. OPG can reduce the production of osteoclasts by

inhibiting the differentiation of osteoclast precursors and by regulating the resorption of osteoclasts. Many studies are in favor of the assertion that the RANKL/OPG ratio is an important determinant of bone mass and skeletal integrity. This study, after the examination of the above bone turnover markers concluded that treatment with enoxaparin decreases the bone ALP concentration, suggesting a possible mechanism of heparin- induced osteoporosis.

Finally, Sarahrudi *et al.* [57] first analyzed the alterations in the expression of osteogenic growth factors in patients with long bone fracture treated with enoxaparin. They measured (M-CSF, VEGF and TGF- β1) after treatment with enoxaparin and they observed significant differences of the expression of growth factors without any influence on fracture healing process.

DISCUSSION

It is difficult to assess the true effect of LMWH on fracture healing process. Based on literature research, there are no studies on the role of LMWHs on fracture healing in humans. The difficulties to evaluate the effects of LMWHs on the skeletal system of humans becomes from two serious reasons. First because the number of patients receiving LMWHs for long periods is limited. Second because experiments on fracture healing in humans contains many risks and complications. For these reasons, the most important evidence for the role of LMWHs on fracture healing comes from *in vitro* and animal studies.

The results of the animal studies (rats, rabbits) vary from no impairment of LMWHs on healing [43-46] to impairment on the healing process [41]. The fracture healing process in animal studies was assessed by histological, radiological and mechanical methods. Histological methods performed in all animal studies. But the gold standard method for evaluating fracture healing process, the mechanical tests, performed in only three studies [41, 44, 64]. In that way, because of the small number of animal studies, the different methodology used and the disagreement in results, no conclusive results can be drawn. Moreover, it has been suggested that animal fracture models do not offer any applicability to human fracture healing process. In most animals the cell biology, biochemistry, healing process and therapeutics needs, differ from those of humans [58].

As it concerns the effect of LMWHs to osteoblasts *in vitro* studies the results are contradictory. Many *in vitro* studies have reported a reduced osteoblast- inhibition by LMWH compared to UFH [31, 49]. On the other side, Muir JM *et al.* demonstrated that both heparin and LMWH had the tendency to decrease bone formation by decreasing osteoblast number and activity, but that only heparin increases osteoclast differentiation and activity [48]. As it concerns the effect of LMWHs to osteoclasts it seems from the limited number of studies that LMWHs increase the number and the activity of osteoclasts, stimulating bone resorption [39]. It is certain that *in vitro* studies have the disadvantage that can only mimic *in vivo* conditions but not entirely describe them.

On the basis of the results from the current study, two other factors seem to play a crucial role on fracture healing process. The hematoma at the inflammatory phase of fracture and the angiogenesis. Street *et al.* [41] found that LMWH increased interfragmentary hematoma. Also in another study Street *et al.* has shown that the high potassium concentration of fracture site hematoma is cytoxic to endothelial cells and osteoblasts [59]. Therefore, increased fracture site hematoma volume may have deleterious effects on fracture healing process. On the other side, it is unclear whether the hematoma improves fracture healing by increasing the supply of osteoprogenitor cells [60, 61].

But not only fracture hematoma can be affected by LMWHs also angiogenesis can be influenced [62]. Vascular endothelial growth factor (VEGF) plays a major role in the process of angiogenesis during the fracture repair especially at the early phase. Norby *et al.* reported that the use of LMWHs suppressed the VEGF- induced angiogenesis [63]. Furthermore, the study of Sarahrudi *et al.* was the first comparative systematic measurement of VEGF serum levels in patients receiving enoxaparin. In this study no significant difference of the VEGF expression was observed [57].

Moreover, effects of LMWHs on mechanical properties of unfractured bones have not been intensively studied. There are only a few experimental reports on their effect on bone strength [55].

In clinical practice, pregnancy is one of the few situations in therapeutics where LMWHs are recommended for prolonged use, but it is not clear from the data if LMWHs are responsible for osteoporosis and osteoporotic fractures in pregnant women. That happens because osteoporosis could arise in pregnant women due to other risk factors including pre- pregnancy low BMI or low dietary calcium intense [15, 23, 25].

Furthermore, it is important to understand that the heterogeneous pharmacologic profile of each LMWH, results from the different methods of manufacturing. The mechanisms responsible for the differences between the effects of different LMWHs could be the result of their different ability to bind different proteins. These proteins in their turn affect bone metabolism in different ways. For sure, these mechanisms need to be elucidated. Most studies in literature review were designed to investigate the effect of only one LMWH. The most studied LMWHs were enoxaparin and dalteparin (Table **5**).

Table 5. Number of studies - type of LMWH used.

LMWH	Number of Studies
Ardeparin	1
Bemiparin	-
Certoparin	3
Dalteparin	12
Enoxaparin	15
Nadroparin	7
Parnaparin	1
Reviparin	-
Tanziparin (Logiparin)	3

Table 6. Studies (LMWHs- fracture healing) model.

Authors	Animal Kind	Animal Number	Fractured Bone	Bony Callus Evaluation Period
Street J [41]	Rabbits	48	Ribs	14 days
Kock HJ [42]	Rabbits	30	Femur condyles	6 weeks
Curcelli EM [64]	Rats	72	Tibial diaphysis	28 days
Erli H [43]	Rabbits	26	Metaphysical fracture on femur	6 weeks
Hak D [44]	Rats	Not mentioned	Femur	6 weeks
Filho S [45]	Rats	22	Diaphysial fracture on femur	28 days
Demirtas A [46]	Rats	32	Femur	3 weeks
Say F [47]	Rats	30	Femur	4 weeks

Furthermore, most studies differ for the animal's kind, number, fractured bone, fracture control and mostly for the bony callus evaluation period (Table **6**). The number of animals is important for estimating the magnitude of the effect of LMWHs. The average number of animals used in these studies was about thirty. For assessing the real effect of LMWHs on fracture healing process large clinical studies are needed, designed to compare different types of LMWHs, different dosages and in different patient groups. Until then no safe conclusions can be made and no effect of LMWH is evidence- based.

Finally, despite there is no statistically significant results for the effects of LMWHs on fracture healing process, with some studies to report deleterious effect, it is our fair evaluation that daily LMWH administration should continue to be the golden rule for prophylaxis of DVT in trauma patients (risk: benefit ratio) [69-71]. Especially in patients with a reduced bone mineral density, e.g. after steroid therapy or because of renal insufficiency, hyperparathyroidism or idiopathic osteoporosis, prophylaxis of thromboembolism with a smaller osteocatabolic potential than heparin is decisive.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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