·Basic Research·

Histopathologic effects of a low molecular weight heparin on bone healing in rats: a promising adjuvant in dacryocystorhinostomy

Mehmet Numan Alp[1], Ozdamar Fuad Oken[2], Mustafa Fevzi Sargon[3], Ahmet Ucaner[2]

[1]Department of Ophthalmology, Numune Training and Research Hospital, Ankara 06100, Turkey
[2]Department of Orthopedics and Traumatology, Numune Training and Research Hospital, Ankara 06100, Turkey
[3]Department of Anatomy, Faculty of Medicine, Hacettepe University, Ankara 06100, Turkey
Correspondence to: Mehmet Numan Alp. Turan Gunes Bulvari, 645. Sokak, Akturk 2 Sitesi, No 24, B Blok, Daire 10, Yukari Dikmen, Ankara 06450, Turkey. mnalp@yahoo.com
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Abstract

• AIM: To investigate the effect of short-term prophylactic dose of a low molecular weight heparin (LMWH) drug on the bone healing process in an animal model simulating the osteotomy obtained in dacryocystorhinostomy.

• METHODS: Forty male Wistar albino rats were divided into 2 groups. Subcutaneous injections of enoxaparin 1 mg/kg (enoxaparin-treated group) and saline solution (control group) were performed once daily for 4d, beginning on the first preoperative day. The osteotomy was created at the femoral diaphysis in all animals by using a Kirschner wire. Each group was further divided into 2 subgroups depending on the timing of the second operation, 14 or 21d following initial osteotomy. Patent osteotomy area on the second and the third weeks in each group were calculated by using a computer software on digital micrographs.

• RESULTS: The patent osteotomy areas at the second and the third weeks were significantly larger in the enoxaparin-treated group than those of the control group (P<0.001 for each time-period). In the control group, the patent osteotomy area at the third week of healing was significantly smaller than that of the second week (P=0.003), whereas there was no significant difference between these two measurements in the enoxaparin-treated group (P=0.185).

• CONCLUSION: Short-term administration of enoxaparin resultes in a significant alteration in bone healing at 14 and 21d after injury. LMWHs can be regarded as promising alternative adjuvants in dacryocystorhinostomy after being evaluated with further clinical and animal studies.

• **KEYWORDS:** dacryocystorhinostomy; enoxaparin; wound healing; low molecular weight heparin
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INTRODUCTION

Dacryocystorhinostomy (DCR) is an important treatment in the relief of tearing and could be performed either through an external or endonasal approach. External DCR has been widely accepted as the gold standard in the treatment of acquired nasolacrimal duct obstruction [1]. With the advent of rigid endoscopes and endoscopic instrumentation, endonasal DCR has become an alternative method to external DCR, but the success rate of this procedure was found to be lower than the conventional method [2-3]. The most frequently reported causes of failure in DCR are obstruction of the common canalicullus and closure of the osteotomy site due to bone regrowth, fibrosis, scarring, and granulation tissue [4]. The idea of inhibiting the causes of failure made the intraoperative application of the antiproliferative agents to be considered as an adjunctive therapy in DCR. Mitomycin-C (MMC) has been the most popular antifibrotic agent used in DCR as well as in pterygium excision and glaucoma surgery with favourable results [5-8]. On the other hand, the presence of well documented ocular complications related to the adjunctive use of MMC in pterygium and filtration surgery [9-10] and the current controversies regarding the optimal dosage and exposure time of MMC [5,7,11] bring considerable limitations about the routine use of this drug in ophthalmic surgery. Normal wound healing process may also interfere with the patency of intranasal ostium after the surgery [8]. Fibrin is accused as a key product in this process that results in granulation and scar tissue formation and eventually bone regrowth [12]. Therefore decreasing or inhibiting fibrin production by pharmacological interruption of the coagulation cascade seems to be a logical and an alternative

method for preventing the aforementioned causes of failure.

Both heparin and its derivative, low molecular weight heparin (LMWH), can successfully inhibit thrombus formation. According to its properties, we feel that LMWHs would theoretically be as effective as the current antiproliferative agents in the prevention of fibrosis, scarring, and bone regrowth at the surgical site following DCR. In this study, we investigated this hypothesis in an animal model concerning the effect of short-term prophylactic dose of a LMWH on the bone healing process in rats.

MATERIALS AND METHODS

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Ankara Numune Training and Research Hospital Ethical Committee and Animal Laboratories of Ankara University Faculty of Medicine. Forty male Wistar albino rats weighing 280-330 g were maintained under controlled temperature, humidity, and light (12h:12h artificial light cycle) conditions for at least 10d prior to study. They were given unlimited access to water and food. On the preoperative day, rats were arbitrarily assigned to 2 groups of 20 animals each and were placed in individual cages. Animals in the enoxaparin-treated group were received 1 mg/kg enoxaparin (Clexane, Aventis Pharma, France) and those in the control group were treated with the same volume of normal saline. The subcutaneous injections were administered once daily to alternate sites of the anterior abdominal wall for 4d beginning on the first preoperative day.

On the operative day, anesthesia was induced using intraperitoneal thiopental at 100 mg/kg body weight. The right femoral bone of each subject was used to prepare experimental fractures and histological specimens. Each animal was placed in the left lateral position; hair on the right thigh widely shaved; and the surgical field was disinfected with povidone iodine solution. Approximately 3 cm of full thickness, longitudinal incision was created to expose muscular fascia and blunt dissection was performed on the muscles lateral to the femoral bone. An osteotomy, simulating the one obtained in DCR surgery, was created at the femoral diaphysis by using a 2.0 mm diameter Kirschner wire (Synthes, Monument, CO, USA). The incision was sutured back in layers with interrupted 4/0 nylon sutures and the sutures were removed on the postoperative day 7.

The enoxaparin-treated group and the control group were further divided into two subgroups each containing randomly chosen 10 animals and reoperated on the second or the third postoperative weeks to prepare the histological specimens. Reoperations were performed under thiopental anesthesia and the animals were sacrificed after complete removal of the previously operated femoral bones. All of the operations were performed by the same surgeon (Oken OF) who was blind to groups. In both groups, there were no adverse events including unusual bleeding during or following the operations.

Histological specimens were prepared including 1 cm of the normal femoral tissue, extending both distally and proximally to the osteotomy site and the callus tissue. All of the light and the scanning electron microscopic examinations were performed by the same doctor (Sargon MF) masked to which group the specimen was assigned.

For qualitative analysis of the osteotomy site by scanning electron microscopic examination; the bone samples were fixed in 2.5% gluteraldehyde for 24h, washed in phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) for 2h and dehydrated in increasing concentrations of acetone. Following the dehydration procedure, the samples were air-dried and they were mounted on metal stubs with a double-sided adhesive band. Then, the specimens were sputtered with a 100 Angstrom thick layer of gold in a BIO-RAD sputter apparatus (England). All the samples were examined with a JEOL SEM ASID-10 Scanning Electron Microscope (Jeol Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

The patency of osteotomy and callus formation on the second and the third weeks following the first operation in the enoxaparin-treated group and the control group were quantitatively assessed by scanning the photographs taken with a Nikon Optiphot light microscope (Tokyo, Japan) into high resolution digital images. The patent osteotomy area was calculated after outlining the callus free area on the scanned micrographs by using an imaging analysis and software program. In order to minimize the observer bias, the image analysis was performed by an examiner masked to which group the micrograph was belonged into.

Statistical Analysis Statistical analyses were performed by using SPSS software (version 15.0, SPSS, Inc.). Results are presented as median (minimum-maximum) values. Mann-Whitney U test was used for evaluation of the statistical significance of difference between the groups with regard to the use of enoxaparin treatment and between the subgroups at respective times, as the second and the third weeks of healing. In each treatment group, one sample t-test was used to compare the changes in the patent osteotomy area measured on the second and the third weeks of healing to a reference value of 3.14-mm^2 that is the area of original hole created at the first operation by a 2.0 mm Kirschner wire. A P-value less than 0.05 was considered statistically significant.

RESULTS

Median values for the patent osteotomy area measured on the second and the third week of healing for the enoxaparin-treated and the control groups are shown in Table 1. Patent osteotomy areas measured both on the second and the

Table 1 The patent osteotomy area of the groups on the second and the third weeks of healing

Groups	Second week of healing (mm^2)	Third week of healing (mm^2)	*P*
Enoxaparin-treated group	0.51 (0.39-0.77)	0.44 (0.30-0.58)	0.185
Control group	0.26 (0.17-0.38)	0.14 (0.04-0.27)	0.003
P	< 0.001	< 0.001	

third weeks of healing were statistically significantly larger in the enoxaparin-treated group (Figure 1) than those of the control group (Figure 2) (Mann-Whitney *U* test, $P<0.001$ for each time period). Similarly, in both the enoxaparin-treated group and the control group, the patent osteotomy areas measured on the second week of healing were found to be larger than those for the third week of healing but the difference was statistically significant only in the control group (Mann-Whitney *U* test, $P=0.003$). In the enoxaparin-treated group and the control group, the patent osteotomy areas measured on the second and the third weeks of healing were found to be statistically significantly reduced as compared to the reference value of 3.14-mm^2 (one sample *t*-test, $P<0.001$ for each time period of each treatment group).

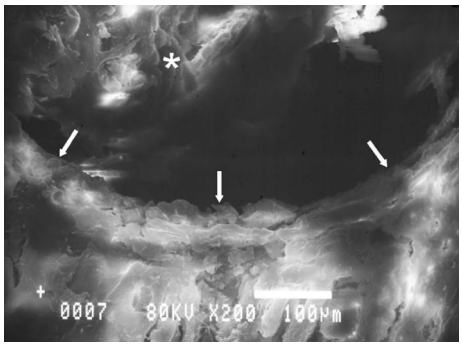

Figure 1 Scanning electron micrograph taken on the third week of healing in the enoxaparintreated group shows a patent osteotomy with smooth edges (arrows) and minimal callus tissue (asterix) (Original magnification ×200).

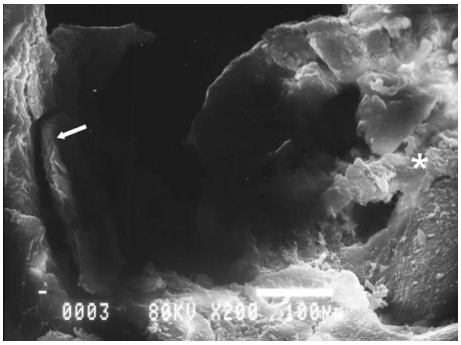

Figure 2 Scanning electron micrograph taken on the third week of healing in the control group shows a significantly occluded osteotomy with ill defined edges (arrow) as a result of extensive callus formation (asterix) (Original magnification ×200).

DISCUSSION

The principle of DCR surgery, either *via* an external skin incision or *via* endonasal approach, is similar to glaucoma surgery. It is to achieve an incomplete healing after DCR surgery to enhance lacrimal outflow into the nose through a patent osteotomy created on the lacrimal bone. Success rates of this procedure have been reported to be over 90% in most of the previous studies [13]. Although it has been generally accepted that an osteotomy size of 15 to 20 mm in diameter is enough to be successful in external DCR, Linberg *et al*[14] showed that the diameter of the healed intranasal ostium was decreased to 1.8 mm, that was only 10% size of the initial surgical ostium, in the postoperative period with excellent functional results [15]. This finding made endoscopic DCR as an alternative method with less disfavors as compared to external DCR. However, the success rate of endoscopic DCR was found to be lower than external DCR [2]. Obstruction of the common canalicullus and closure of the rhinostomy site due to the consequences of normal wound healing pathways, such as bone regrowth, fibrosis, scarring, and granulation tissue have been reported as the most common causes of DCR failure [3-4,13]. Serial biological events leading to tissue repair occur following a surgical injury to the tissue. Replacement processes take place in the initial phase of repair and involve inflammation and coagulation cascades that ultimately lead to scar tissue formation. On the other hand, regeneration processes result in a variable degree of restoration of the original tissue structure and formation of the scar tissue depending on the regeneration capacity of the injured tissue. Inflammation and scar tissue formation can be subsided by taking some meticulous precautions, such as preoperative evaluation and management of intranasal abnormalities, prevention of thermal damage associated with the excessive use of lasers and drills, or avoiding unnecessary trauma through gentle handling of tissue during surgery. However, other causes associated with the normal wound healing may also interfere with the success of DCR. According to Lama and Fechtner [16], normal wound healing cascade can theoretically be modulated at several stages by using different pharmacological agents interfering with particular factors involved in the pathway. They simply classified these agents into four groups with regard to the main phases of repair: coagulative, inflammatory, proliferative, and post-proliferative remodeling. Many authors, for example, recommend the intraoperative application of MMC to the surgical anastamosis as an antiproliferative agent in this manner [7-8]. MMC is an alkylating agent that inhibits fibroblast proliferation at the rhinostomy site with favourable outcomes. However, its use

is advocated especially in the setting of a failed DCR because of its potential for significant ocular and intranasal complications [9]. Therefore, there have been some efforts to find a substitute for MMC in prevention of surgical failure.

Pharmacologically interrupting the coagulation cascade at an earlier step could theoretically modulate the wound healing response and prevent the closure of surgical osteotomy. Zilelioglu *et al* [8] reported that the decrease in the size of the healed intranasal ostium after surgery was the result of a normal wound healing response. Wound healing may be considered as one of the activities of hemostasis, which is initiated by direct tissue injury and blood vessel disruption as a result of surgical manipulation. Fibrin is the end product of the coagulation process and is formed by conversion of fibrinogen to fibrin by thrombin. Fibrin is responsible for a series of histochemical reactions, such as platelet aggregation, proliferation and synthesis of extracellular matrix, that eventually result in granulation and scar tissue formation[12].

Both heparin and its derivative, LMWH, can successfully inhibit thrombus formation. Heparin has some limitations based on its pharmacokinetic, biophysical, and biological properties not shared by LMWHs[17]. LMWH preparations are produced by chemical or enzymatic depolymerization of native heparin and are considered to be more convenient in the prophylactic inhibition of fibrin formation. The studies concerning the efficacy and safety of LMWHs have shown that LMWHs equally prevent deep venous thrombosis and pulmonary embolism and result in significantly lesser bleeding complications when compared to unfractionated heparin and warfarin [18]. LMWHs do not ineract with platelet function and do not modify bleeding time, thus, requiring less intense laboratory monitoring. Although LMWHs are mainly used for thromboprophylaxis in orthopedic surgery, they have been also found to be safe and effective in the prevention of ocular scarring and neovascularization disorders by reduction of cell-mediated contraction and cellular proliferation[18-19].

The subject of the current study is mainly based on the clinical and experimental observations elucidating the side effects of LMWHs on bone healing process. Long-term uses of heparin and LMWHs have been shown to carry a risk of osteoporosis as a biological limitation [20-22]. Standard heparin has been reported to decrease bone formation and increase bone resorption [22]. Although, there are a number of studies reporting a similar or a lesser effect of LMWHs on the skeletal system, the exact mechanism of LMWHs on bone healing when used with a standard dosage for thromboprophylaxis have not been fully understood[20-23]. In an unstabilized rabbit rib fracture model, Street *et al* [24] has suggested that a short-term administration of LMWH thromboprophylaxis would delay bone healing by two distinct mechanisms, either by disrupting the formation of osteoprogenitor units or by increasing bleeding tendency that would cause interfragmentary hematoma collection. Although it is generally accepted that the fracture site hematoma plays a beneficial role in fracture healing, they reported that an increased amount of fracture site hematoma comprises high potassium concentration that is cytotoxic to endothelial cells and osteoblasts and may display negative effects on bone formation during fracture healing that could be accepted as a welcome effect for DCR. As a consequence of these observations related with the short-term use of LMWHs in clinical practice of orthopedic surgery, this study was initiated to investigate the potentially beneficial effect of short-term prophylactic dose of a LMWH on the bone healing process in an animal model, in which a Kirschner wire was used to create an osteotomy with a similar diameter to the healed intranasal ostium obtained after DCR with sufficient functional results[14-15].

The present study revealed that, although, the patent osteotomy area decreased after two weeks and three weeks in all of the animals treated with or without enoxaparin, the decrease was statistically significant only in the control group. Moreover, the patent osteotomy areas measured both on the second and the third weeks of healing remained significantly larger in enoxaparintreated animals when compared to those of the controls. This observation seems to support our hypothesis that an exposure to enoxaparin may affect early bone healing and may alter the closure of osteotomy site in the early postoperative period following DCR. On the other hand, we feel that there are some limitations to the routine use of LMWHs in DCR as an adjunctive drug. The main limitation is probably an increased tendency for intraoperative and postoperative bleeding related with DCR in patients receiving LMWH. It has been suggested that these drugs are discontinued if possible upon consultation with the patient's primary care physician. On the other hand, there are reports suggesting that endoscopic DCR is a safe and efficient treatment for relief of distal nasolacrimal duct obstruction in anti-coagulated patients as compared to patients without anti-coagulation therapy and does not require discontinuing their anti-coagulant therapy, including warfarin and coumadin [25-26]. We suggest that choosing the most effective and the safest administration route (systemic *vs* topical), dosage, and exposure time of LMWH are the other issues related with the adjunctive use of these drugs in DCR surgery remain to be investigated with further studies.

The current study we presented is a preliminary animal study and shows that short-term administration of a low dose of enoxaparin resulted in a significant alteration in bone healing with less callus formation at 14 and 21d after injury. Our results suggest that LMWHs could be regarded as promising alternative adjuvants in DCR surgery. We also believe that

additional clinical and animal studies regarding the aspects so far mentioned are required to assert the routine use of LMWHs in DCR surgery and to compare these drugs with currently used antiproliferative agents.

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