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Is enoxaparin sodium exactly safe for subcutaneous fibroblast?: A cell culture study

Enoksaparin sodyum subkutan fibroblast için tam olarak güvenli midir?: Bir hücre kültürü çalışması

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ABSTRACT

Aim: Despite relatively low amount in the subcutaneous tissue, fibroblasts play a critical role in the continuity of intercellular connections, maintenance of tissue integrity, and forming a balanced fascial network. Enoxaparin sodium is widely used in the prophylaxis and treatment of deep vein thrombosis. In the present study, we aimed to examine the cytotoxic effects of enoxaparin sodium on fibroblast cells in an in vitro model.

Material and Methods: L929 mouse fibroblast cells were treated with enoxaparin sodium 4000 IU, 2000 IU, 1000 IU, 500 IU, and 250 IU. At 24 hours, cell morphology was evaluated; cell viability was analyzed through methylthiazole tetrazolium assay and propidium iodide/ acridine orange staining was made to support changes in nuclear morphology as the sign of initial apoptosis.

Results: The test results showed that high doses of enoxaparin sodium (4000 IU, 2000 IU) exerted cytotoxic effects and induced apoptotic morphology in initial stage. Compared to the control group, there was no significant difference in the cell viability in Dilutions III, IV, and V.

Conclusion: Based on our results, despite prophylactic dose in the in vitro setting, high-dose enoxaparin showed cytotoxic effects. Long-term high-dose enoxaparin sodium may affect the number of subcutaneous fibroblasts, impairing the skin integrity and subcutaneous tissue healing

Keywords: Enoxaparin sodium, cytotoxicity, apoptosis, skin, fibroblast.

ÖΖ

Amaç: Fibroblastlar, subkutan dokuda nispeten düşük miktarlara rağmen, hücreler arası bağlantıların sü-rekliliğinde, doku bütünlüğünün korunmasında ve deri fasyası ile dengeli bir iletişim oluşturmada kritik rol oynarlar. Enoksaparin sodyum, derin ven trombozunun profilaksisinde ve tedavisinde yaygın olarak kullanılmak-tadır. Bu çalışmada, enoksaparin sodyumun fibroblast hücreleri üzerindeki sitotoksik etkilerini in vitro bir mod-elde incelemeyi amaçladık.

Materyal ve metod: L929 fare fibroblast hücreleri enoksaparin sodyum 4000 IU, 2000 IU, 1000 IU, 500 IU ve 250 IU ile işleme tabi tutuldu. 24 saatte hücre morfolojisi değerlendirildi; hücre canlılığı metiltiyazol tetrazolyum deneyi ile analiz edildi ve propidium iodide/acridine orange boyamasıyla apoptozisin başlangıcını gösteren nükleer değişiklikler incelendi.

Bulgular: Enoxaparin sodyumun yüksek dozlarının (4000 IU, 2000 IU) sitotoksik etkiler yarattığı görüldü. Kontrol grubu ile karşılaştırıldığında, Dilüsyon III, IV ve V'de hücre canlılığında istatistiksel açıdan anlamlı bir fark bulunmadı.

Sonuç: Sonuçlarımıza göre, in vitro ortamda profilaktik doza rağmen, yüksek doz enoksaparin sitotoksik ve apoptotik etkiler göstermiştir. Uzun süreli yüksek doz enoksaparin sodyum, deri altı fibroblastların sayısını etkileyerek deri bütünlüğünü ve deri altı doku iyileşmesini bozabilir.

Anahtar Kelimeler: Enoksaparin sodyum, sitotoksisite, apoptoz, deri, fibroblast.

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INTRODUCTION

ow-molecular-weight heparins (LMWHs) are chemical or enzymatic depolymerization of unfractionated heparins (UFHs) with an average weight of 5,000 Da [1]. Unlike UFHs, short chains of LMWHs are less interacted with plasma proteins, directly inhibiting factor Xa [2]. In addition, as LMWHs exert less effects on platelets, the incidence of thrombocytopenia with LMWHs is relatively low than UFHs [2,3]. Since LMWHs do not contain long polysaccharide chains which inhibit factor IIa, they do not alter the partial thromboplastin with a very high absorption with subcutaneous administration, thereby, leading to a bioavailability ratio of up to 90% [2]. As the behavior of LMWHs following absorption is more predictable than UFHs, monitoring is not necessary. Although LMWHs can be administered intravenously similar to UFHs, high bioavailability ratio makes them suitable for subcutaneous administration, as well. In particular, enoxaparin sodium is an effective and safe first generation LMWH for the prevention and treatment of venous thromboembolism and for the prevention of mechanical heart valve thrombosis, as well as in specific patient populations including pregnant and cancer patients [4]. Alt-hough LMWHs indicated as safe drugs, LMWHs may lead to injection site problems such as irritation, pain, bruising, redness, and swelling. In addtion, severe skin reactions related to enoxaparin sodium can be found in the literature [5-7]. Delayed hypersensitivity reactions in the injection site are the most commonly reported reactions, although systemic reactions are rarely reported [6,7].

Although fibroblasts are overlooked due to their low amount in the subcutaneous tissue, they play a critical role in the continuity of intercellular connections, maintenance of tissue integrity, and forming a balanced fascial network, preserving the skin elasticity [8,9]. In addition, fibroblasts are essential for epithelial cell differentiation, inflammation processes, and wound healing, as they are mesenchymal-derived cells [10]. However, there is no study in the literature investigating the effect of subcutaneous enoxaparin injections on fibroblasts.

In the present study, we hypothesized that

enoxaparin sodium, a LMWH, might exert toxic effects on the fibroblast cells in subcutaneous tissue. Therefore, we aimed to examine the cytotoxic effects of enoxaparin sodium on subcutaneous fibroblast cells, which are the first encountered cells in the subcutaneous tissue, in a cell culture model.

MATERIALS AND METHODS

There is no data in the literature which indicates the exact number of fibroblasts in subcutaneous tissue. However, this might be estimated according to histological preparations. In the present study, we created a cell culture model based on histology preparations [11]. There are approximately 16 fibroblast cells were counted in a 0.02 mm² subcutaneous section. It was estimated that there were 76,000 cells in 1 cm² area in vivo. In this study, 96-well plate (Greiner Bio-One, Germany) was used for fibroblast cell culture. Area of each well is 0.32 cm². Therefore, we found that we need to put 25000 fibroblast cells in each well in vitro.

Cell culture and treatment of cells with test material

L929 mouse fibroblast cells were cultured in a 96well plate (Greiner Bio-One, Germany) and 25,000 cells/mL in each six replicate plates were seeded. The cells were, then, incubated in Dulbecco's Modi-fied Eagle's Medium (DMEM)/Ham's F12 (Biowest Inc., Nuaillé, France) containing 10% fetal bovine serum (FBS) (Biowest Inc., Nuaillé, France) at a humid environment and 95% air and -5% CO2 and 37°C for 12 hours. After incubation, the cells were treated with five dilutions of the test material [Clexane[™] (enoxaparin sodium, 4,000 IU/0.4 mL). Dilutions were prepared in the medium as Dilution I: 4,000 IU; Dilution II: 2,000 IU; Dilution III: 1,000 IU; Dilution IV: 500 IU; and Dilution V: 250 IU. In the control group, the cells were incubated in the test material-free medium.

Assessment of cell morphology and viability

We described assessment of cell morphology and viability our previous studies [12,13]. Briefly, Altera-tions in the cell morphology were analyzed using an inverted microscope (IX70 Olympus, Japan). At 24 hours, all dilutions treated with the test material were compared with the control group. The cell viability was analyzed through (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) (MTT) assay. At 24 hours of incubation, the media were removed and 12.5 μ L MTT solution (Sigma-Aldrich, Germany) was added into 100 μ L FBS-free DMEM/F12 for the each well. The cell culture plates were wrapped with aluminum foil and incubated for four hours. Then, MTT solution was removed and 100 μ L isopropyl alcohol (Amresco Inc., USA) was added to discontinue reaction. The cell viability was measured through an ultraviolet (UV)-visible spectrophotometer (EZ Reac 400 Microplate Reader, Biochrom) at an absorbance of 560 nm wavelength.

In the in vitro setting, the cytotoxic effects of enoxaparin sodium were investigated. Baseline number of L929 cells was calculated as 25,000 in each well (25,000 cells/0.32 cm²/96-well) (Corning Inc., NY, USA). The viability ratios of cells (%) were calculated according to the MTT results.

Acridine orange/propidium iodide staining was made for staining dead cells with degenerated nucleus to support cell viability [14]. At 24 hours of incubation, the media on the cells were removed and AO/PI (Sigma-Aldrich, Germany) was added without fixation at a v/v ratio of 1:1 and incubated for 20 sec. Subsequently, the cells were washed with phosphate buffer saline (PBS) (Sigma-Aldrich, Germany) for 10 sec and covered with a PBS: glycerol (v/v: 1:1) mounting medium. The cells were, then, examined under a florescence microscope. Dead cells were evaluated by counting red cells with fragmented nuclei. The AO/PI-stained cells were observed under a narrow band fluorescein (FITC) filter (520-560 nm) in green color, and PI-stained cells were observed under rhodamine filter (510-560 nm) as stained red.

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). Descriptive statistics were expressed in mean ± standard deviation (SD). The Kolmogorov-Smirnov test was used for the normality test. The analysis of variance (ANOVA) was used to compare the means of more than two groups. A post-hoc test (least significant difference [LSD] or Tamhane's test) was used to analyze significant differences among the groups. A p value less than 0.05 was considered statistically significant.

RESULTS

Assessment of cell morphology and viability

At 24 hours of incubation, there was a significant degeneration different from normal fibroblast morphology in the Dilutions I and II treated with enoxaparin sodium, compared to the control group. These cells were round shaped rather than normal fibroblast morphology (i.e., elongated cells) with dense nuclei (Fig 1A and B). When Dilution I was compared with Dilution II, there was a higher rate of degeneration in Dilution I. However, Dilutions III, IV, and V showed normal fibroblast morphology with elongated and spindle-shape patterns. Similar to the control group, the cells completely covered the culture dish and became confluent in all three Dilutions (Fig 1C, D, E, and F).



Figure 1. Morphological appearance of L929 mouse fibroblasts exposed to enoxaparin sodium Dilution I (A) (20×); Dilution II (B) (10×); Dilution III (C) (10×); Dilution IV (D) (10×); Dilution V (E) (10×); and Control (F) (10×) at 48 hours of incubation (black arrows indicate rounded and degenerated cells).

The results of the MTT assay are shown in Table 1. Compared to the control group, Dilution I showed the highest cytotoxic effect, followed by Dilution II (P=0,001, P=0,029). Compared to the control group, there was no significant difference in the cell viability in Dilutions III, IV, and V (P>0.05). Accordingly, the cell viability decreased almost 50% in Dilutions 1 and 2 at 24 h of incubation with high-dose enoxaparin sodium. This finding indicated death of about half of subcutaneous tissue fibroblasts per 1 cm². (Table 2)

Table 1. MTT results of enoxaparin sodium at 24 hours in each dilution compared to control group

Time	Dilutions	Mean	sd	Р
24h	D1	0,157	0,045	0,001
	D2	0,213	0,040	0,029
	D3	0,275	0,110	0,648
	D4	0,307	0,038	0,815
	D5	0,316	0,053	0,413
	Control	0,318	0,134	-

Mean: Absorbance (OD), sd: standart deviation, *P values compared to control group. Dilution I: 4000 IU; Dilution II: 2000 IU; Dilution III: 1000 IU; Dilution IV: 500 IU and Dilution V: 250 IU.

Table 2. Comparison of cell viability in in vitro and in vivo settings at 24 hours in each dilution

Dilutions	Cell Viability (%)	Percentage of rounded dead cells
		with AO/PI staining (%)
D1	42	51
D2	67	29
D3	87	18
D4	97	5
D5	99	2

Dilution I: 4000 IU; Dilution II: 2000 IU; Dilution III: 1000 IU; Dilution IV: 500 IU and Dilution V: 250 IU. Initial cell numbers: (25.000 cells/0.32 cm2/in vitro)

Based on AO/PI staining, the cells were assessed according to two main criteria: Normal fibroblast cells elongated with intact nuclei and were stained with green color, while dead cells were in red dye with round-shape morphology and fragmented nuclei. According to these criteria, the cytotoxic effect was prominent in Dilution I (Fig 2A). In Dilution II, there were rounded dead cells (Fig 2B). In Dilutions III, IV, and V, there was no significant alteration in the cell morphology, compared to the control group (Fig 2C, D, and E).



Figure 2. AO/PI staining of L929 mouse fibroblasts exposed to enoxaparin sodium. Red and round dead cells in Dilution I (A) (40×); Red and round dead cells and green healthy and normal cells in Dilution II (B) (40×); Green healthy and normal cells in Dilution III (C) (40×), Dilution IV (D) (40×), Dilution V (E) (40×) and Control (F) (40×) at 24 hours of incubation.

DISCUSSION

The subcutaneous tissue is the innermost layer of the skin between the dermis and fascia. It is composed of subcutaneous fat, connective tissue, and lobules of fat. The thickness and components of the subcutaneous tissue vary depending on the site of the body and collagens and elastic fibers produced by the tissue fibroblasts play a key role in the maintenance of the integrity and shape of the cells and continuity of a balanced interaction between the cells and fascia and undermost muscle layers [8]. Fibroblasts are mesenchymal-derived cells with a spindle-shaped morphology and non-epithelial, endothelial, or immunological cells (in turn, cytokeratin; E-cadherine; 'CD31-' and 'CD45-') [15]. They are responsible for tissue homeostasis in normal physiological circumstances [16]. In addi-tion, fibroblasts release extracellular matrix (ECM) proteins, preserving the continuity of the matrix. In case of tissue injury, these cells are activated and differentiated into myofibroblasts which contract and participate in healing by reducing

the wound size and secreting ECM proteins [17]. As fibroblasts in the reticular dermis, hypodermal (subcutaneous) fibroblasts play a central role in wound healing [17]. Although fibroblasts are in a metabolic resting state in healthy tissues, they are activated and release chemockines, cytokines, and growth factors as well as ECM proteins, thereby, contributing to the healing process in case of tissue injury [15]. Although the number of fibroblasts is relatively low in the subcutaneous tissue than the other tissues, they are essential in wound healing. There is no data in the literature which indicates the exact number of fibroblasts in subcutaneous tissue. In our study, we evaluated healthy dermal histologic preparations and have created a cell culture model according to our evaluation results.

In case of any damage in the fibroblasts, cellular morphology and intercellular connections and signal conduction become impaired. Previous studies demonstrated that repetitive subcutaneous injections impaired morphology of fibroblasts and produced an elongated shape than a spindle shape [8]. In an acupuncture study, repetitive rotations of acupuncture needles led to a similar result, depending on the intensity of the application [9]. Of note, these studies discussed mechanical or physical effect-induced the alterations in the connective tissue fibroblasts. However, there is still no study investi-gating the direct effect of agent which is subcutaneously administered on fibroblasts. In an animal model, toxic effects of subcutaneous enoxaparin injection were evaluated using systemic parameters in mice and no direct cytotoxic effects on fibroblasts in the subcutaneous tissue were observed [18]. In the present study, we observed that cytotoxicity of enoxaparin sodium of the subcutaneous fibro-blast cells increased dependent with initial exposure doses. In addition, it was found that high dose of enoxaparin sodium was associated with high cell lost.

Enoxaparin sodium is subcutaneously administered generally through the anterolateral abdominal wall by altering the administration site in each attempt between the left and right anterolateral ab-dominal wall [8]. Several factors including injection site, duration and volume of the medication and the needle size play a role in the development of injection site reactions. The most common complications associated with LMWHs including enoxaparin sodium are delayed hypersensitivity reactions, eczema, and plaque formation in the injection site [19]. In particular, the most common delayed hypersensitivity reactions in the injection site include itchy erythema, infiltrated plaques, vesicular or bullous plaques, and necrotic plaques, while eczema, maculopapular exanthema or acute generalized exanthematous pustulosis can be secondarily seen [20].

The severity of skin reaction may vary depending on the age and sex of the patient, the presence of pregnancy or obesity. Obesity is an important factor for the dose adjustment of the medications used for prophylaxis or treatment of venous thromboembolism [21,22]. Although guidelines for the dose adjustment of enoxaparin sodium for overweight or obese patients have been published, the initial dose should be modified according to the body weight of an individual patient. Consequently, high dose enoxaparin is required in obese patients [23]. When the dose is uptitrated based on the body weight, the amount of enoxaparin exposure to the fibroblasts also increases, thereby, inducing cytotoxic effect of the drug. However, the cytotoxic effects of the medication itself have not been investigated in these studies. Our study results showed that high-dose enoxaparin sodium induced loss of nearly half of the cell viability. In the in vivo setting, this finding indicates death of about half of subcutaneous tissue fibroblasts per 1 cm². The doubling time of human fibroblasts is 24 h which is longer than the doubling time of L929 cells (16 h) in the in vitro model [24]. This suggests that regeneration is longer in the in vivo setting and repetitive drug administration may lead to irreversible subcutaneous tissue damage. The absorption of enoxaparin sodium is directly dose-dependent and is immediately and totally absorbed following the subcutaneous injection with a bioavailability of nearly 100% [25]. Accordingly, high-dose enoxaparin is needed to achieve an immediate bioavailability in the in vitro setting; however, it is associated with increased cytotoxic effects in the injection site (as shown in Dilution I).

Nonetheless, there are some limitations to this study. First, the L929 mouse fibroblasts were used

instead of human fibroblasts. However, these fibroblasts have been widely used in cytotoxicity studies, being a well-defined cell line. Their shorter doubling time than human fibroblasts is another advantage of these cells in the in vitro setting. Second, this study used a two-dimensional in vitro model. Further studies using three-dimensional models would contribute to the existing literature and support our findings.

Conclusion: In conclusion, despite prophylactic dose in the in vitro setting, high dose enoxaparin showed cytotoxic effects. Based on these results, long-term high dose enoxaparin sodium may affect the number of subcutaneous fibroblasts, impairing the skin integrity and subcutaneous tissue healing. However, further studies are needed to gain a better understanding of the clinical and subclinical effects of cytotoxicity of LMWH in subcutaneous fibroblasts.

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