TJVS Turkish Journal of Vascular Surgery

Turk J Vasc Surg 2024;33(2):59-63
DOI: 10.9739/tjvs.2024.03.013

Available online at www.turkishjournalofvascularsurgery.org

Original Article

An investigation of the dose-dependent safety of bemiparin sodium: A cell culture study

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Received: March 29, 2024 Accepted: May 15, 2024 Published online: July 01, 2024

Abstract

Aim: Fibroblasts are common cells in subcutaneous tissue and they have an important role on healing process. Although patients who are receiving first-generation low molecular weight heparins are more likely to experience skin responses, there is no study evaluating the effect of bemiparin sodium on subcutaneous fibroblasts following administration. In the present study, we aimed to evaluate the effects of bemiparin sodium on fibroblast cell viability in cell culture model.

Material and Methods: The L929 cells were incubated for 12 hours in 96-well culture dishes. Fresh medium containing different concentrations of bemiparin was added in five different concentrations (Dilution I: 3,500 IU; Dilution II: 1,750 IU; Dilution III: 875 IU; Dilution IV: 437.5 IU; Dilution V: 218.75 IU). A control group was established with drug-free culture medium. Cell viability analysis was performed after 48 hours of incubation by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. The change in cell morphology was examined at each dilution by direct and acridine orange/propidium iodide staining.

Results: The highest cytotoxic effect was observed in dilution I compared to the control group ($p<0.05$). There is a clear deviation from the fibroblastic morphology of the cells in dilution I. The cells have a round morphology with degeneration and nuclear condensation.

Conclusion: Although it seems that high doses of bemiparin may have a negative effect on fibroblast cells in subcutaneous tissue, it can be considered that the cytotoxic effects of high concentrations of the drug at the application site will not be clinically significant due to using a single dose per day.

Keywords: Bemiparin, cell culture techniques, fibroblasts, subcutaneous tissue

INTRODUCTION

Bemiparin sodium is a low molecular weight heparin (LMWH) molecule obtained by chemical or enzymatic depolymerization of unfractionated heparin (UFH), an anticoagulant [1]. LMWHs are recognized as effective and safe anticoagulants in the treatment and prevention of acute venous thromboembolism (VTE) due to their high bioavailability [2-4]. Bemiparin sodium has a molecular weight of 3600 daltons and one mg of bemiparin sodium inhibits approximately 80-120 IU of Factor Xa. The anti-factor Xa/anti-factor IIa ratio is approximately 8 and the half-life is longer than other LMWH molecules [5]. This allows them to be administered as a single daily dose. While LMWHs can be administered intravenously, bemiparin sodium is often administered subcutaneously due to its high bioavailability [5].

In addition to side effects such as bleeding, thrombocytopenia, systemic allergic reaction, hyperkalaemia and osteoporosis, heparin may cause allergic or non-allergic skin lesions [6-8]. Because skin lesions are often associated with the route of administration, patients receiving LMWHs are at increased risk for skin reactions. First-generation LMWHs have been reported to cause skin lesions. However, there are very few case reports of second-generation LMWHs and no studies investigating

CITATION
Kubat E, Gurpinar OA, Demirkiran T. An investigation of the dose-dependent safety of bemiparin sodium: A cell culture study. Turk J Vasc Surg. 2024;33(2):59-63.

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the effect of bemiparin sodium on subcutaneous fibroblasts after administration. Therefore, the objective of this study was to evaluate the effects of bemiparin sodium on fibroblast cell viability.

MATERIAL AND METHODS

Cell Culture and Treatment of Cells with Test Material

In this study, the L929 cell line was used to investigate the cytotoxic effect of bemiparin. The L929 cell line is a well-characterized cell line isolated from mouse subcutaneous connective tissue in continuous culture (American Type Culture Collection (ATCC)) and is widely preferred for cytotoxicity assays. In our study, Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (Biowest Inc., Nuaillé, France) containing 10% fetal bovine serum (FBS) (Biowest Inc., Nuaillé, France) was used for the assay with 25,000 cells per well in 96-well culture dishes. After incubation at 95% air-5% CO2 and 37°C for 12 hours under standard culture conditions, the medium was removed and fresh medium containing different concentrations of bemiparin was added. Since the test material is water soluble, it was prepared as a stock solution of bemiparin sodium (HiborTM) (3,500 IU/0.2 ml) in culture medium in five different concentrations (Dilution I: 3,500 IU; Dilution II: 1,750 IU; Dilution III: 875 IU; Dilution IV: 437.5 IU; Dilution V: 218.75 IU). A control group was established with drug-free culture medium. The experimental design was designed to perform six replicate analyses.

Assessment of Cell Viability and Cell Morphology

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) analysis

Cell viability analysis was performed after 48 hours of incubation by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) method [9]. For this purpose, the culture medium on the cells was removed 48 hours later and incubated with 10% MTT solution (Sigma-Aldrich, Germany) prepared in serum-free medium for 4 hours in the dark. At the end of this time, the MTT solution was removed, the reaction was stopped by the addition of isopropyl alcohol, and cell viability was measured as the absorbance value at 560 nm (EZ Reac 400 Microplate Reader, Biochrom).

Acridine orange/Propidium iodide (AO/PI) staining

The change in cell morphology was examined at each dilution by direct and AO/PI staining. For AO/PI staining, the medium was removed after 48 hours of incubation and AO/PI (Sigma-Aldrich, Germany) was added to the cells at a 1:1 (v/v) ratio without fixation. After 20 seconds, the dye solution was removed and washed with phosphate buffer saline (PBS) (Sigma-Aldrich, Germany) for 10 seconds. Mounting medium prepared as PBS:glycerol (v/v: 1:1) was added to cover the cell surface. Cell morphology was examined by evaluating the fluorescence color change (IX70 Olympus, fluorescence attachment, Japan).

Statistical Analysis

Statistical analysis was performed using IBM SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). Descriptive statistics are expressed as mean±standard deviation (SD). The Kolmogorov-Smirnov test was used to test for normality. 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 between groups. A p-value less than 0.05 was considered statistically significant.

RESULTS

Assessment of Cell Viability and Cell Morphology

MTT analysis

The results of the MTT assay are shown in Table 1. Accordingly, the highest cytotoxic effect was observed in dilution I compared to the control group ($p<0.05$). When dilutions II, III, IV and V were compared with the control group, cell viability was similar to the control group and cell viability was similar between groups ($p>0.05$).

Table 1. MTT results of enoxaparin sodium at 48 hours in each dilution compared to control group

Time	Dilutions	Cell viability (OD value)	Min-max values	P
48 h	I	0.075	0.064-0.08	<0.001
	II	0.219	0.213-0.227	0.06
	III	0.221	0.217-0.227	0.09
	IV	0.228	0.212-0.244	1
	V	0.229	0.218-0.235	1
	Control	0.231	0.224-0.241	-

Mean: Absorbance (OD), sd: standart deviation, *P values compared to control group
I: 3.500 IU; II: 1.750 IU; III: 875; IV: 437,5; V: 218,75 and Control

AO/PI staining

The change in cell morphology when viewed directly with an inverted microscope is shown in Figure 1.

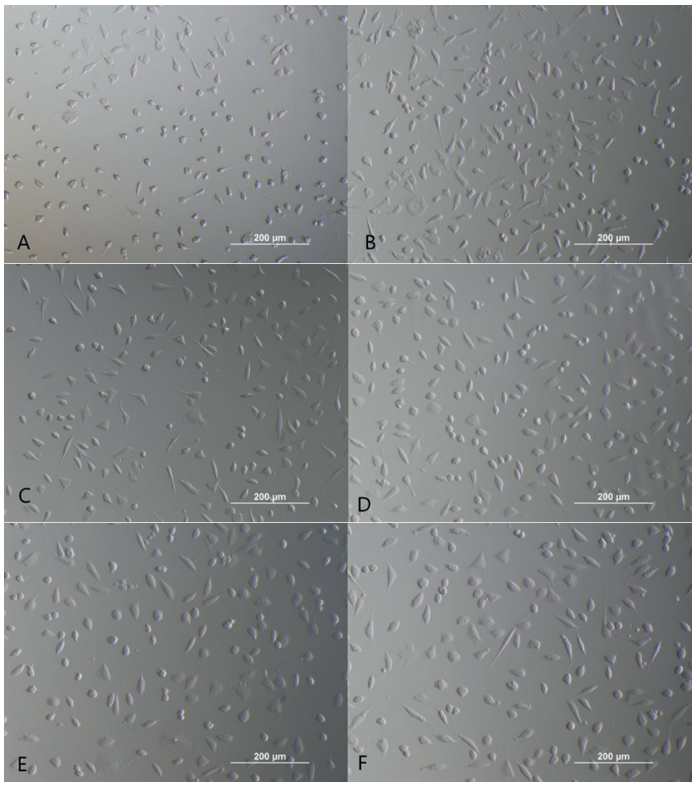

Figure 1. Morphological appearance of L929 mouse fibroblasts exposed to bemiparin sodium Dilution I (**A**) (10×); Dilution II (**B**) (10×); Dilution III (**C**) (10×); Dilution IV (**D**) (10×); Dilution V (**E**) (10×); and Control (**F**) (10×) at 48 hours of incubation

As shown in Figure 1, there is a clear deviation from the fibroblastic morphology of the cells in dilution I. The cells have a round morphology with degeneration and nuclear condensation. In dilution II, cells with a similar morphology were observed, although very rarely. In dilutions III, IV and V, the cell morphology was similar to healthy cells and similar to the control group.

Two types of cell morphology have been defined in the evaluation of AO/PI staining. Normal morphology cells are stained green and have an intact nucleus, whereas dead cells with apoptotic morphology have a yellow-orange-red, rounded morphology and a fragmented nucleus (Figure 2).

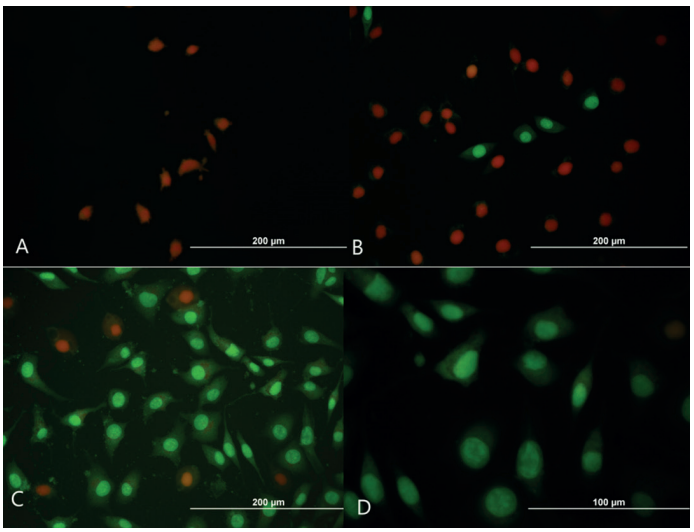

Figure 2. AO/PI staining of L929 mouse fibroblasts exposed to enoxaparin sodium. Red and round apoptotic cells in Dilution I (**A**) (20×); Red and round apoptotic cells and green healthy and normal cells in Dilution III (**B**) (20×); Green healthy and normal cells in Dilution V (**C**) (20×) and Control (**D**) (20×) at 48 hour

The percentage of apoptotic cells is shown in Table 2. These results are consistent with the results of the MTT assay; the highest percentage of apoptotic cells is observed at dilution I. At other dilutions, the percentage of apoptotic cells was similar to the control group.

Table 2. The ratio of apoptotic cells at 48 hours in each dilution and control group

Time	Dilutions	Percent of apoptotic cells	Percent of normal cells	P
48 h	I	98	2	<0.001
	II	4.2	95.8	0.06
	III	3.5	96.5	0.09
	IV	3.1	96.9	1
	V	3.2	96.8	1
	Control	2.9	97.1	-

DISCUSSION

Although bemiparin is an LMWH formed by depolymerization of UFH, unlike UFH, it binds less to platelet factor 4 (PF4) protein [1]. PF4 is a heparin-bound protein that exerts an anti-thrombin effect by inhibiting anti-thrombin reactions with thrombin and Factor X. It is also responsible for neutralizing heparin sulphate, a glycosaminoglycan found on the cell surface. As a result,

bemiparin has far fewer systemic side effects than UFH [1]. It also has higher bioavailability and long-term anticoagulant activity.

Subcutaneous tissue is the inner layer of the skin and consists of adipose tissue and connective tissue elements. It is also rich in blood vessels and nerves [10,11]. Its thickness varies according to the area of the body where it is located, and both its thickness and distribution vary from person to person and gender to gender. Subcutaneous tissue is the loose connective tissue that connects the skin to the underlying structures and gives it the ability to move over the parts of the body to which it is attached [10-12]. Adipocytes and fibroblasts are common cells of loose connective tissue. Fibroblasts are responsible for the production of collagen and elastin, and the resulting interaction with the extracellular matrix ensures the integrity of the tissue [10]. Fibroblasts, which are mesenchymal cells of connective tissue, show heterogeneity and form responses by differentiating or secreting different factors according to the signals they receive from their microenvironment [13]. Fibroblasts of subcutaneous tissue are reticular fibroblasts and differentiate into fibroblasts responsible for the production of fibrillar extracellular matrix and preadipocytes and adipocytes [14]. These cells, which are in a dormant state in healthy tissues, become active and participate in the healing process through the action of various chemokines, cytokines and growth factors that are released when tissue damage occurs [13]. Therefore, any morphologic and metabolic damage to these cells or disruption of intercellular interactions may lead to disruption of the wound healing process in the skin. However, studies have examined the effects of various LMWHs directly on endothelial cells and related angiogenic processes. Güler et al. investigated the cytotoxic effect of UFH and LMWH on human umbilical vein cells [15]. In their study, the effects of the same doses of bemiparin, nadroparin, enoxaparin and heparin on the viability of endothelial cells (human umbilical endothelial vein, HUVEC) were examined and the highest cytotoxic effect was observed in the group treated with bemiparin sodium [15]. However, there are also studies in the literature that discuss different results.

With repeated applications of LMWH, various reactions at the application site have been reported, depending on the time and site of application or the type of needle [13]. These are often delayed hypersensitivity reactions, erythema, or necrotic plaque formation. It is also emphasized in case reports that more serious skin reactions may be shaped according to the sex, age, presence of diabetes and obesity or pregnancy status of the person [13]. Val et al. published an exceptional case report of a subcutaneous infraumbilical abdominal nodule which was 3.8 cm in diameter, solitary, fibrofatty and which had atypical (with bizarre nuclei) cells with poorly defined borders in a 23-year-old patient 4 years after local LMWH (enoxaparin sodium, 40 IU sc/24 h) use [16]. When the retrospective LMWH history of this patient was reviewed, they found that high doses of medication were used and emphasized in their article that "As the clinical history of injections is often not reported on the requisition form, pathologists should be aware of this adverse effect of LMWHs to avoid misdiagnosis and overtreatment" [15]. Although bemiparin sodium, the lowest molecular weight LMWH molecule used in our study, is an effective and safe agent in the treatment of VTE, it may cause mild bleeding or heparin-associated thrombocytopenia [17]. It has also been reported that skin swelling and erythema or local eczematous reactions at the injection site may be observed 24-72 hours after administration, although less than other LMWHs [18].

However, as noted above, there are studies reporting that first-generation LMWHs cause skin lesions [19,20]. However, there are very few case reports of second-generation LMWHs and no studies investigating the effect of bemiparin sodium directly on subcutaneous fibroblasts after application. According to our results from this study, which we designed to investigate this effect, high doses of bemiparin were cytotoxic under in vitro conditions. This suggests that long-term use of bemiparin may have a negative effect on fibroblast cells in subcutaneous tissue. However, this relationship needs to be confirmed by further in vitro and in vivo studies.

Our study has several limitations. First, our study is an in vitro study using a two-dimensional power line. The cell line used instead of human fibroblasts is the L-929 mouse subcutaneous connective tissue-derived fibroblast cell line. Although the L-29 cell line is one-third the size of human fibroblasts, its characteristics are very similar to human fibroblasts [13]. It is also a well-characterized cell line for L-929 mouse cell cytotoxicity assays and is a suitable cell line for in vitro applications as its doubling time is twice as fast as that of human fibroblasts. In conclusion, VTE is an important health problem and effective and safe anticoagulant therapy is of paramount importance. Bemiparin sodium is a good option at this time. However, the results of our study showed that high doses of bemiparin were cytotoxic under in vitro conditions. This suggests that long-term use of bemiparin may have a negative effect on fibroblast cells in subcutaneous tissue. However, this relationship needs to be confirmed by further in vitro and in vivo studies.

CONCLUSION

According to our results from this study, high doses of bemiparin were cytotoxic under in vitro conditions. Although it seems that high doses of bemiparin may have a negative effect on fibroblast cells in subcutaneous tissue, it can be considered that the cytotoxic effects of high concentrations of the drug at the application site will not be clinically significant due to using a single dose per day. However, this relationship needs to be confirmed by further in vitro and in vivo studies.

Ethics Committee Approval: This study is an in vitro cell culture study. No patient samples were used. Therefore, ethics committee permission and patient consent form are not required.

Patient Consent for Publication: This study is an in vitro cell culture study. No patient samples were used. Therefore, ethics committee permission and patient consent form are not required.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: All authors contributed equally to the article.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding: The authors received no financial support for the research and/or authorship of this article.

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