Türk Göğüs Kalp Damar Cerrahisi Dergisi 2017;25(2):264-272
http://dx.doi.org/doi: 10.5606/tgkdc.dergisi.2017.13700

Experimental Study / Deneysel Çalışma

The effect of bemiparin on neointimal hyperplasia and endothelial cell proliferation in a rabbit carotid artery model

Tavşan karotis arter modelinde bemiparinin neointimal hiperplazi ve endotelyal hücre proliferasyonu üzerine etkisi

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ABSTRACT

Background: The aim of this study was to investigate the effects of bemiparin on neointimal hyperplasia and endothelial cell proliferation in a rabbit carotid artery model.

Methods: Between March 2015 and April 2015 12 randomly-selected New Zealand male rabbits, weighing 2 to 3 kg, were divided into two groups. The right carotid arteries were transected and sutured with 8-0 polypropylene material with continuous suture technique. The control group (n=8) received no additional medication, while the study group (n=6) received 150 IU/kg/day of bemiparin for seven days. All rabbits were sacrificed on Day 28, and the carotid artery segments were removed and prepared for histological examination.

Results: All histochemical and histomorphological analyses were performed by two investigators who were blind to the groups. In the cross-section analysis of the vessel specimens of the control group, thickening of the tunica intima was noticed, and in a section, the intimal thickening was almost occluding the lumen with a few recanalization areas. In the bemiparin group, the intimal hyperplasia ($p<0.006$) and the thickness of the tunica media decreased ($p<0.018$), compared to the control group. There was no significant difference in the histomorphometric analysis results of the mean luminal diameters and luminal areas between the groups ($p<0.100$, $p<0.068$, respectively).

Conclusion: Our study results suggest that bemiparin exerts its effect preventing neointimal hyperplasia and endothelial cell proliferation in animal model.

Keywords: Aorta; bemiparin; carotid artery; intimal hyperplasia; vascular endothelial growth factor.

ÖZ

Amaç: Bu çalışmada bemiparinin neointimal hiperplazi ve endotelyal hücre proliferasyonu üzerine olan etkileri bir tavşan karotis arter modelinde araştırıldı.

Çalışma planı: Mart 2015 - Nisan 2015 tarihleri arasında rastgele seçilen, 2 ila 3 kg ağırlığında 12 Yeni Zelanda tavşanı iki gruba ayrıldı. Sağ karotis arterler transekte edildi ve 8-0 prolen, devamlı sütur tekniği ile sütüre edildi. Kontrol grubuna (n=6) herhangi bir ilaç verilmez iken, çalışma grubuna (n=6) yedi gün boyunca 150 IU/kg/gün bemiparin verildi. Tavşanların tümü 28. günde sakrifiye edildi ve karotis arter segmentleri çıkarılarak, histolojik inceleme için hazırlandı.

Bulgular: Tüm histokimyasal ve histomorfolojik analizler gruplara kör iki araştırmacı tarafından yapıldı. Kontrol grubunun damar örneklerinin kesitsel analizinde, tunika intimada kalınlaşma izlendi ve bir kesitte intimal kalınlaşma çok az sayıda rekanalizasyon alanları ile birlikte lümeni neredeyse tamamen tıkamıştı. Bemiparin grubunda, kontrol grubuna kıyasla, intimal hiperplazi ($p<0.006$) ve tunika media kalınlığı ($p<0.018$) azalmıştı. Gruplar arasında ortalama luminal çapları ve luminal alanlarının histomorfometrik analizi açısından anlamlı bir fark yoktu (sırasıyla $p<0.100$, $p<0.068$).

Sonuç: Çalışma sonuçlarımız, hayvan modelinde bemiparinin neointimal hiperplazi ve endotelyal hücre proliferasyonunu önleyerek etkisini gösterdiğini ortaya koymaktadır.

Anahtar sözcükler: Aort; bemiparin; karotis arter; intimal hiperplazi; vasküler endotelyal büyüme faktörü.

Available online at
www.tgkdc.dergisi.org
doi: 10.5606/tgkdc.dergisi.2017.13700
QR (Quick Response) Code

Received: July 14, 2016 *Accepted:* October 15, 2016

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Impairment of vascular endothelial integrity is on the basis of arterial thrombosis. Accordingly, it continues to be promoted with thrombosis.[1-3] There are various intraoperative medical agents administered to suppress this process, including heparin, warfarin, low-molecular-weight heparin (LMWH), dextran, acetylsalicylic acid, and prostaglandins. In clinical practice, LMWH is known to be successful for the prevention and treatment of thromboembolism.[1,2]

Potent anti-thrombotic agents, wide therapeutic index, lower bleeding risk, cost-effectiveness, a favorable risk-benefit ratio, and patient compliance and satisfaction are critical factors for choosing a LMWH molecule. Lower bleeding risk (2%), predictable responses, longer duration of half-time, lower incidences of bone loss, and lower allergic reaction and bacterial contamination are the main advantages of LMWHs.[2,3] In addition, they have been reported to be more useful, compared to unfractioned heparins (UFHs).

In recent years, ultra-LMWHs (ULMWHs less than 5.000 Da) have been developed to improve the efficiency of anti-thrombotic treatment of occlusive arterial diseases. However, the protection mechanism responsible for this effect has not been elucidated, yet. Establishment of a carotid artery model in animals would be, hence, useful for better understanding the immunohistochemistry in human patients.

Bemiparin is a ULMWHs.[3-5] Nowadays, it is licensed for using thromboprophylaxis after either general or orthopedic surgery. In this study, we aimed to investigate the effects of bemiparin on intimal hyperplasia and endothelial cell proliferation in a rabbit carotid artery model.

MATERIALS AND METHODS

This randomized, controlled, interventional experimental study was conducted at the Animal Laboratory and Research Center of Dokuz Eylül University, Faculty of Medicine with the approval of the Ethics Committee for Animal Research on 16 December 2014 with the protocol number 67/2014. All procedures were performed according to the principles of the Laboratory Animal Care by the National Institutes of Health. Care was given to the animals on a 12-h dark/light cycle at temperature (24±2°) and humidity (55-60%), and was maintained on ad libitum throughout the experiment.

The experiment was performed on 12 male rabbits (2,000 to 3,000 g; age 10 to 12 weeks) according to the guidelines provided by the experimental animal laboratory. Animals were divided into two groups with six subjects in each group. All animals in the bemiparin group received bemiparin 150 IU/kg (Hibor®, Rovi Pharmaceutical Laboratories, Madrid, Spain; batch 11105A) for seven days. The control group did not receive any prophylactic treatment. Right-sided carotid arteries were transected and restored with 8-0 polypropylene sutures (Figures 1, 2). Bemiparin was injected subcutaneously according to the dose

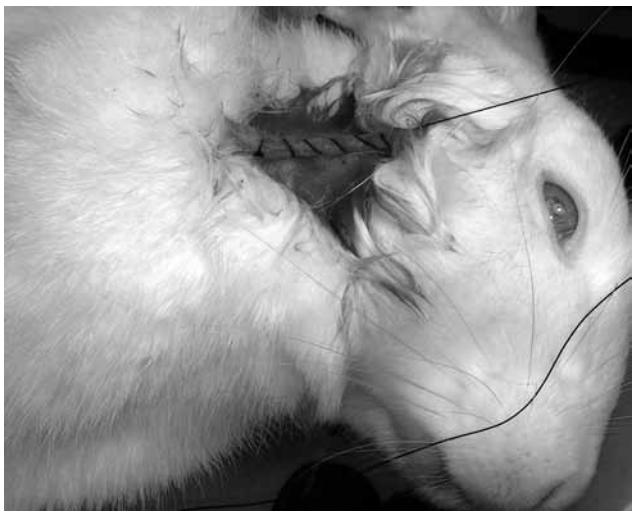

Figure 1. Animal model image 1.

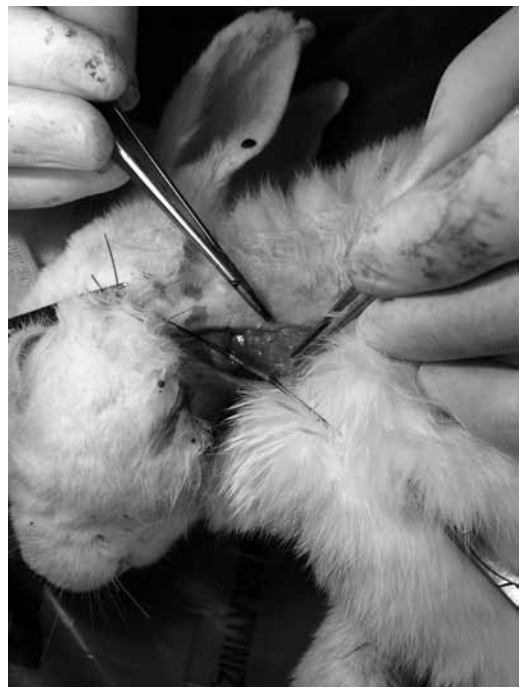

Figure 2. Animal model image 2.

used in previous studies in the literature.[6] All rabbits were sacrificed on Day 28, and the carotid artery segments were removed and prepared for histological specimens.

Anesthesia and surgical procedure

Intramuscular anesthesia was admininistered using ketamine hydrochloride 50 mg/kg (Pfizer, İstanbul, Turkey) and xylazine 5 mg/kg (Bayer Türk Kimya Sanayi, İstanbul, Turkey). Cefazolin sodium 50 mg/kg (Bilim İlac Sanayi, İstanbul, Turkey) intramuscularly was used to prevent infections. In addition, 3.5x surgical loupes (Designs for Vision Inc., Long Island, NY, USA) were used during dissection and operation. All animals were first placed in supine position. The right carotid arteries were reached near the trachea and excised about 1 cm in size for the operation. Next, a 1 mm in length incision was made in the blocked interval of the artery with a silk suture to its vertical axis, using an iris blade for coronary surgery. The right carotid artery segment was sutured with continuous suture technique using a 6.5 mm, 3-8 circle, 8-0 polypropylene material (Ethicon Inc., Somerville, NJ, USA). Maintenance of arterial blood flow during the procedure was achieved with this technique. The surgical procedures are shown in Figures 1 and 2. Skin closure was performed with 2-0 polypropylene (Johnson & Johnson Medical Devices Companies, Belgium). The animals were, then, kept on a 12-h dark/light cycles at temperature (24±2 °C) and humidity (55-60%), and were maintained on ad libitum throughout the experiment. The euthanasia was performed with high-dose ketamine and xylazine, and the carotid artery specimens were taken during surgery for morphological examinations on postoperative Day 28.

Histopathological evaluation

Light microscopic examination

The carotid arteries were removed and fixed in 10% neutral-buffered formalin, dehydrated in a graded series of isopropyl alcohol (60 to 100%), followed by xylol before being embedded in paraffin. After specimens were blocked in paraffin, 5 μm sections were taken with rotary microtome (RM 2255, Leica Instruments, Nussloch, Germany) on poly-L lysine coated slides. The hematoxylin and eosin (H&E, Surgipath, 01562E, Bretton, Cambridgeshire, UK)-stained sections were used to evaluate overall histomorphological and morphometric measurements. Images were obtained from the selected areas and analyzed using a computer-assisted image analyzer system consisting of a microscope (Olympus BX-51, Tokyo, Japan) equipped with a high-resolution video camera (Olympus, DP70, Japan). All measurements were made using the UTHSCSA Image Tool version 3.0 software (University of Texas Health Science Center, San Antonio, TX, USA). The luminal diameters, luminal areas, intima, media thickness were all measured and compared between the groups.

Immunohistochemical examination

Immunohistochemical staining was performed using the streptavidin-avidin-biotin method. Sections of 5 μm were taken with a rotary microtome (RM 2255, Leica Instruments, Nussloch, Germany) and were incubated at 60 °C overnight and, then, dewaxed in xylene for 30 min. After rehydrating through a decreasing series of alcohol, the sections were washed in distilled water for 10 min. They were, then, treated with 10 mM citrate buffer (Cat No.AP- 9003-125 Labvision) at 95 °C for five min to unmask antigens by heat treatment. The slides were, then, rinsed three times for two min each with deionized water. All sections were delineated using a Dako pen (Dako, Glostrup, Denmark) and incubated in a solution of 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. They were, then, incubated with normal serum blocking solution for 30 min and were incubated in a humid chamber overnight at 4 °C with (endothelial nitric oxide synthase [eNOS], anti-eNOS mouse monoclonal Ab, Genetex, USA). Alpha-smooth muscle actin (α-SMA) (ab7817, Abcam) primer antibodies. They were washed three times for five min each with phosphate-buffered saline (PBS), followed by incubation with biotinylated immunoglobulin G (IgG) and, then, with streptavidin-peroxidase conjugate (Invitrogen, Histostain-Plus Kit Broad Spectrum, 85-9043). After washing three times for five min with PBS, the sections were incubated for five min with a 3.3'-diaminobenzidine (DAB) (1718096, Roche, Germany) to detect immunoreactivity and, then, counterstained with the Mayer's hematoxylin. The sections were covered with Entellan® (Merck, KGaA, Darmstadt, Germany) and were observed by light microscopy using a BH-2 microscope (Olympus, Tokyo, Japan).

Image analysis methods

A computerized video camera-based image analysis system (UTHSCSA Image Tool software version 3.0, University of Texas Health Science Center, San Antonio, TX, USA) was used. All available sections (at least three sections per vessel) were analyzed; only sections with obvious technical artefacts related to the staining procedure were excluded. After the staining process completed, the

sections were examined under a light microscope (Olympus BX-50 Tokyo, Japan) and images transferred to computer using a high-resolution camera (Olympus DP-70, Tokyo, Japan). All sections were digitally captured.

Semi-quantification of immunostaining data

A grade system was used to score the quantity of e-NOS and α-SMA immunopositive staining. The score was defined as the following: 1, very few positive staining was observed in an image and the staining was mild; 2, positive staining was moderate and between grade 1 and grade 3; 3, strong positive staining was evenly distributed in the whole image, and 0, no immunoreactivity. To maintain consistency of scoring, each section was graded by two investigators who were blinded to the groups and the mean values were calculated. Digital microscopic images were taken at the area, where the positive cells were observed for each vessel section. The mean scores were used to represent the grade of e-NOS and α-SMA staining for each vessel.

Statistical analysis

Direction and significance of the association between non-parametric variables were evaluated by using Fisher's exact test. In all calculations and statistical analyses, the SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and Excel software (Microsoft Inc., Redmond, WA, USA) were used. Semi-quantitative immune scoring variables were expressed in mean ± standard deviation (SD). A p value of less than 0.05 was considered statistically significant.

RESULTS

In the cross-sections of the control group vessel samples, thickening of the tunica intima was noticed; in one case, the intimal thickening was almost occluding the lumen with a few recanalization areas. In addition, there was an increase in the thickness of the tunica media and a prominent thickening in the adventitia in this group (Figure 3). The mean intima thickness was 14.2 μm (range, 10.44 to 18.57 μm) in the bemiparin group (Table 1), and 53.0 μm (range, 34.07 to 81.72 μm) in the control group (Table 2). The mean media thickness was 115.8 μm (range, 82.9 to 160.66 μm) in the bemiparin group and 167.6 μm (range, 133.74 to 221.82 μm) in the control group. In the bemiparin group, the intimal hyperplasia ($p<0.006$) and the thickness of the tunica media decreased ($p<0.018$),

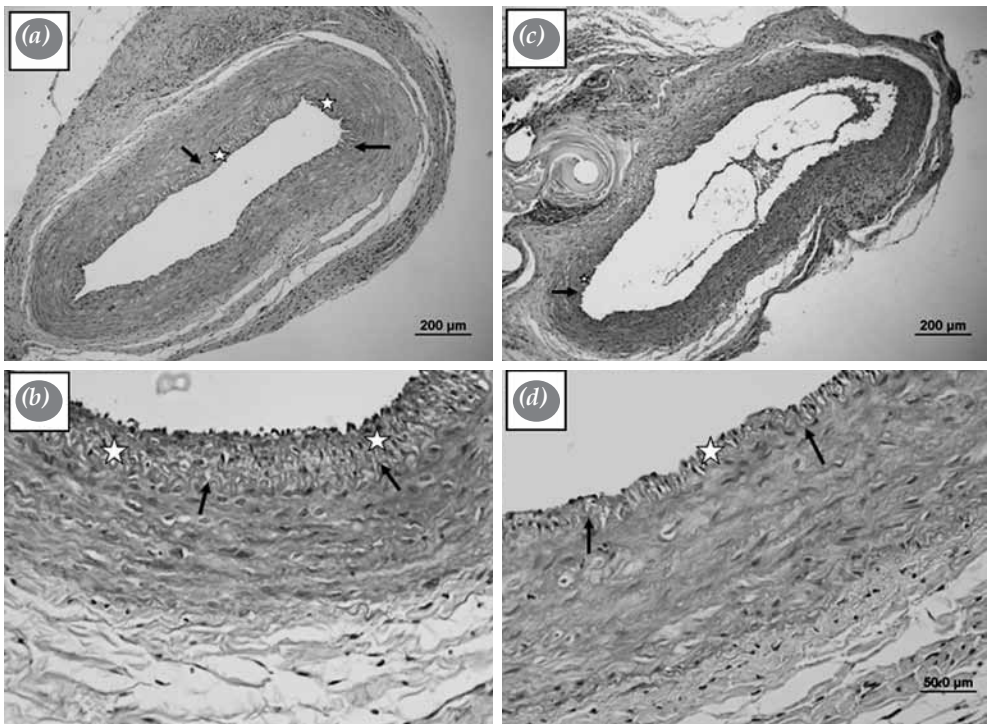

Figure 3. Histological sections of groups, control and bemiparin. **(a, b)** Control group. **(c, d)** Bemiparin group (H-E x 40).

★ Internal elastic lamina; → Intimal hyperplazia.

Table 1. Mean lumen diameters and lumen area and mean intima-media area values in bemiparin group

	Valid	Missing	Median	Mean±SD
Lumen diameter (μm)	5	0	577.19	630.7±175.8
Luminal areas (μm^2)	5	0	242349.54	242396.4±89998.7
Intima (μm)	5	0	13.12	14.2±3.3
Media (μm)	5	0	102.36	115.8±30.5

SD: Standard deviation.

compared to the control group. The mean lumen diameter was 630.7 μm (range, 453.20 to 894.35 μm) in the bemiparin group and 770.6 μm (range, 659.75 to 981.10 μm) in the control group. The mean lumen area was 242396.4 μm^2 (range, 188311.4 to 675472 μm^2) in the bemiparin group and 167.6 μm^2 (range, 142604.2 to 380269 μm^2) in the control group. The mean luminal diameters and luminal areas of the experimental groups were all evaluated by histomorphometry and no significant difference was found between them ($p<0.100$, $p<0.068$), respectively (Figure 4). Scoring the quantity of e-NOS and α-SMA-positive staining revealed a non-significant difference between the experimental groups (based on the Kruskal-Wallis test, $p<0.05$).

DISCUSSION

The present study demonstrated the effects of bemiparin on the cellular and ultrastructural changes and intimal hyperplasia in a model of rabbit carotid artery by histomorphological, immunohistological, and ultrastructural examinations.

The choice of medical agents is critical in the presence of vascular restenosis or in complex pathological settings, such as arterial thrombosis, where the atherosclerotic plaque plays a fundamental role. Arterial thrombosis following coronary artery bypass grafting is a major clinical problem. Recent studies have suggested the use of of LMWHs to reduce the risk of restenosis.[3-5] In clinical practice, these agents have been replaced among other anticoagulants as the standard choice of therapy.[5,7-10] Due to existing evidences, we hypothesized that bemiparin could affect neointimal hyperplasia and endothelial cell proliferation. The present study was, therefore, addressed into the effects of bemiparin upon the establishment of an animal carotid artery model. The experimental study model was performed by surgical intervention of carotid artery, followed by transection and suturation techniques.

Bemiparin is a LMWH and has shown beneficial effects in the thromboprophylaxis and the treatment of deep vein thrombosis.[11-15] It is known as a second-generation LMWH due to its ultra-low molecular weight (3.600 Da). According to its low molecular weight, it has lower anti-factor IIa (thrombin) activity and an anti-Xa: anti-IIa activity ratio of 8:1 versus a ratio of 1:1 for UFHs and dalteparin 2-3:1.[3,4]

In today's practice, LMWHs have replaced standard heparin in daily clinical setting. They have a high bioavailability (90%) independent from the dose with a two to four hours half-time.[3-6] Elimination is through renal pathway. Also, heparins with shorter chains have improved bioavailability and slower clearance. Their anticoagulant effects are with the inhibition of factor Xa and anti-thrombin. They have lower incidence of bleeding and side effects rather than UFHs. In addition, LMWHs are successful in the prevention and treatment of thromboembolism.[6,13-25] They are more effective than UFH in venous thromboembolism (VTE) and non-Q wave myocardial infarction. Routine heparin therapy in this setting is still under debate. Furthermore, LMWHs are more effective than standard heparin when added to thrombolytic agents; however, bleeding risk increases.[3-6]

On the other hand, the effects on platelet functions of LMWHs and UFH in ischemic conditions is still

Table 2. Mean lumen diameters and lumen area and mean intima-media area values in control group

	Valid	Missing	Median	Mean±SD
Lumen diameter (μm)	6	0	745.14	770.6±108.9
Luminal areas (μm^2)	6	0	347678.94	385592.6±173181.7
Intima (μm)	6	0	52.35	53.0±23.6
Media (μm)	6	0	166.24	167.6±30.5

SD: Standard deviation.

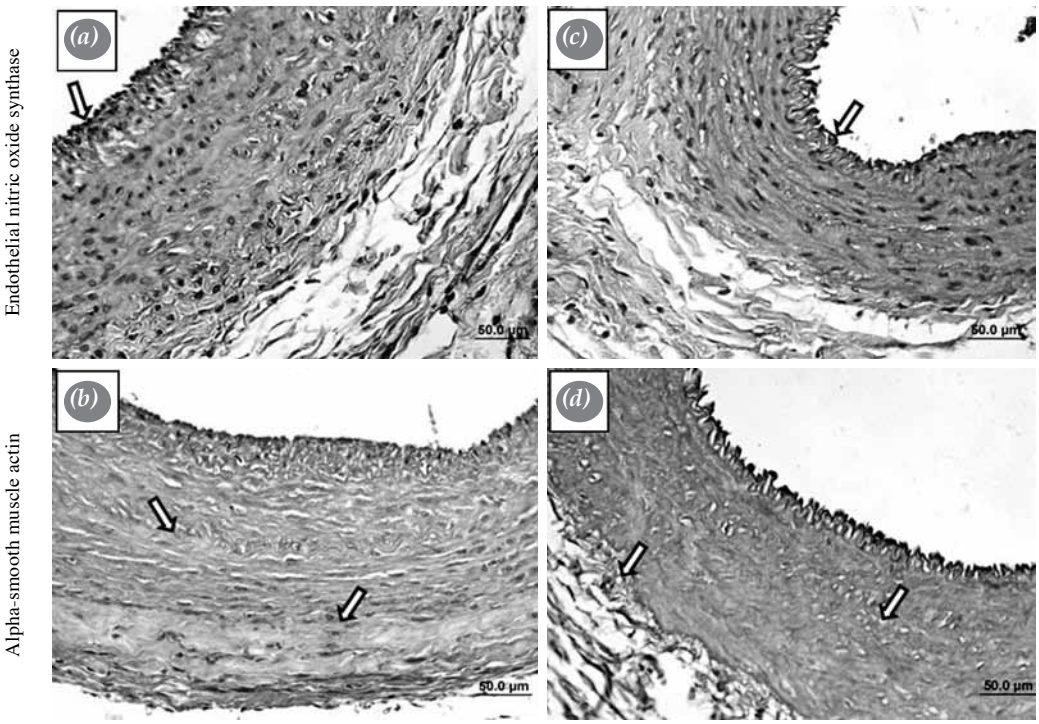

Figure 4. Histological sections of groups, control and bemiparin. **(a, b)** Control group. **(c, d)** Bemiparin group (H-E x 40).
⟹ Endothelial nitric oxide synthase and alpha-smooth muscle actin staining.

under debate and have variations. Both decrease the percentage of subendothelial matrix coating of platelets, and this effect is more prominent in LMWHs.[4,5] Two factors are responsible for this: platelet activation results in factor IV release and thrombogenesis induces factor Xa, thereby, activating the platelets. Also, LMWHs have more affinity to factor Xa.[6-8] This supports LMWHs for protection against arterial thrombosis (i.e., acute coronary syndrome and ischemic stroke).

In the literature, data on outcomes of the effect of bemiparin on intimal hyperplasia and endothelial cell proliferation are limited.[16-21] Da Pozzo et al.[3] reported that bemiparin provided a significant decrease, as shown by the reduction of endothelial cell tubule network, while both fondaparinux and UFH did not show any significant effect on *in vitro* angiogenesis. One major finding of this study is that bemiparin was the only drug to show inhibited angiogenesis *in vitro* experiments. These results are of utmost importance for the selection of a medical therapy. Yavuz et al.[24] also compared anti-angiogenic potentials of rivaroxaban, enoxaparin and tinzaparin sodium in chick chorioallantoic membrane in an *in vivo* experiment. Enoxaparin and tinzaparin dependently increased the anti-angiogenic effects, but did not exceed the median score, whereas rivaroxaban exceeded this threshold and showed more efficacy. In another study, Katrancioglu et al.[25] compared UFH, enoxaparin, and tinzaparin with a similar study protocol. They concluded that UFH had more prominent anti-angiogenic potential than of enoxaparin and tinzaparin. However, the anti-angiogenic effect of tinzaparin was dose-dependent and it was more protective for cardiovascular diseases. Dogan et al.[26] also found evident anti-angiogenetic effects of LMWHs with a similar study protocol. However, they concluded that further investigations should be revealed to detect the difference between the effects of LMWHs.

Sánchez-Ferrer et al.[4] demonstrated that bemiparin increased the release and activity of tissue factor pathway inhibitor (TFPI) from endothelial cells under both static conditions and arterial sheer stress. Depending on the 1 to 2-h earlier peak activity and longer duration of TFPI than the anti-Xa effect, bemiparin showed a successful anti-thrombotic activity and improved pharmacological profile, compared to UFHs. Also, bemiparin had a successful anti-thrombotic activity and a better pharmacological profile than UFHs.[3]

Furthermore, several clinical investigations have been preformed on the pharmacovigilance of bemiparin. The percentage of hematoma in the injection sites is lower with bemiparin.[4,5] Postoperative bemiparin use in neuroaxially anesthetized cases has also lower risk of spinal hematoma.[4,5] It is effective as warfarin in VTE cases for three months and replaces in many cases oral antiocoagulants in clinical practice.[4-6]

In another study, Ferdows et al.[21] suggested dose reduction for prophylactic use of enoxaparin, bemiparin, and certoparin in patients with a creatinine clearance value below 30 mL/min. In addition, they suggested that prophylactic doses of tinzaparin and dalteparin were likely to be safe in patients with renal insufficiency and these patients do not need dose reduction. In previous studies, LMWHs with the lowest molecular weight (enoxaparin, bemiparin, certoparin, and nadroparin) all showed accumulation in a therapeutic and prophylactic dose.[17-21] In addition, Da Pozzo et al.[3] reported that each LMWH was a pleiotropic biological agent with its own chemical, biochemical, biophysical, and biological characteristics, displaying a unique pharmacodynamic and pharmacokinetic profile. According to this study, the safety and efficacy of bemiparin, endowed of the highest anti-FXa/anti-FIIa activity ratio of any second-generation LMWH, were demonstrated. These characteristics are also important to improve the anti-thrombotic profile of an agent.

Caliskan et al.[23] compared the effects of enoxaparin, bemiparin, and rivaroxaban in an ischemia reperfusion model in 40 rats. They measured nitrogen oxide (NOx), prolidase and malondialdehyde (MDA) plasma levels following peripheral ischemia and reperfusion (I/R). The authors proved the prophylactic and therapeutical effects of factor Xa inhibitors against reactive oxygen species (ROS), which lead to ischemia reperfusion injury in post-thrombotic conditions. They concluded that rivaroxaban, a novel oral factor Xa inhibitor, had similar antioxidant protective effects. Their results support that LMWHs decrease oxidative stress and inflammation. In another study, Demirtas et al.[11] investigated whether anticoagulant and antiaggregant agents had protective effects against oxidative damage induced by I/R.[11] Nitrogen oxide levels, MDA levels, paraoxonase-1 (PON-1) activity, and prolidase activity were evaluated in both cardiac and renal tissues. One major finding of this study was that thromboprophylactic agents appeared to provide partial or prominent protection against I/R injury.

In this content, the aim of our study was to evaluate the effects of bemiparin on the cellular and ultrastructural changes in the carotid artery anastomosis model in animals. At the end of the experiment, carotid tissues were removed and fixed. After routine histochemical and histomorphological analyses, tissue sections were investigated immunohistochemically (e-NOS and α-SMA), and ultra-structurally. The former, eNOS, is known to be primarily responsible for the generation of NO in the vascular endothelium.[22] The latter, α-SMA, is known to play a crucial role in fibrogenesis and correlates with the activation of myofibroblasts.[8] Also, SMA is an important part in fibrogenesis and myofibroblast generation.[8] The endothelium plays a major role in regulating usual blood vessel physiology. Intimal hyperplasia is an independent risk factor for cardiovascular field. As a result, arterial elasticity and the muscle smooth muscle cell, collagen, elastic fibrils, and proteoglycans in the matrix alter and vessel wall injury occurs.[8] Many surgeons avoid operating cases with I/R injury related intimal damage, due to disastrous complications. In the present study, we used ULMWHs to reduce the activation of complements and I/R injury-related coagulopathy. We found that, in the bemiparin group, the intimal hyperplasia ($p<0.006$) and thickness of the tunica media (arrows) decreased ($p<0.018$), compared to the control group (Figure 1a, b). Besides, the mean luminal diameters and luminal areas of the experimental groups (arrows) were all evaluated by histomorphometry. However, no significant difference was found between them ($p<0.100$, $p<0.068$), respectively (Figure 2a, b). Scoring the quantity of e-NOS and α-SMA-positive staining revealed a non-significant difference between the experimental groups (based on the Kruskal-Wallis test, $p<0.05$). According to these findings, we believe that bemiparin has prominent protection against neointimal hyperplasia and I/R injury, and is also protective against oxidative stress.

In addition, Pérez-Ruiz et al.[16] compared the effects of bemiparin and an UFH on plasminogen activator inhibitor-1 (PAI-1), tissue-plasminogen activator (t-PA), tissue factor (TF), tissue factor pathway inhibitor release, and PAI-1 gene expression by human umbilical vein endothelial cells. They reported that bemiparin, in contrast with UFH, did not induce an increase in the TF, indicating that bemiparin may be an additional favorable feature.

Study limitations

The major limitation of this study was the lack of molecular data. However, in the future, we plan to perform a study on a higher budget by attaching immunohistochemistry data and oxidative stress parameters.

In conclusion, according to the findings of our study, anti-thrombotic treatment with bemiparin can prevent the development of harmful effects in the vessels. Cardiovascular diseases have complex ultra-structural and multi-functional changes in the vasculature. Intimal hyperplasia, restenosis, and endothelial proliferation processes precedes and elicits vascular damage. Although several animal models have been developed to reveal the mechanisms involved in anti-thrombotic and protective effect of bemiparin, further human studies are needed to assess the prophylactic profile of bemiparin and all ultra low-molecular-weight heparins in patients undergoing peripheral artery and coronary artery disease. Based on our study results, we concluded that bemiparin has superior pharmacological profile compared to other second-generation low-molecular-weight heparins. We consider that it has prophylactic and/or therapeutic effects against reperfusion injury by reducing the oxidative stress.

Declaration of conflicting interests

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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