



Regular Article

Antiangiogenic activities of bemiparin sodium, enoxaparin sodium, nadroparin calcium and tinzaparin sodium

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ABSTRACT

Introduction: The low-molecular-weight heparins have been demonstrated to have antiangiogenic effects in various assays. We aimed to demonstrate and compare the antiangiogenic effects of four types of commercially available low-molecular weight heparins in the chick embryo chorioallantoic membrane model.

Materials and methods: The antiangiogenic efficacies of bemiparin, enoxaparin, nadroparin, and tinzaparin were examined in vivo in the chick chorioallantoic membrane model. Drug solutions are prepared in three different concentrations (100 IU, 10 IU, or 1 IU/10 µl). For each set of experiment twenty fertilized eggs were used. The decrease of vessel formation is examined and scored according to previous literature.

Results: Bemiparin, enoxaparin, nadroparin, and tinzaparin sodium all have antiangiogenic effects on chick chorioallantoic membrane at the concentration of 100 IU/10 µl. This effect was also observed in 10 IU/10 µl concentrations of nadroparin and tinzaparin.

Conclusions: The low molecular weight heparins studied have obvious antiangiogenic effects. There may be a difference in the potency of the drugs that could have a significant implication for further clinical research.

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Introduction

The increased risk of venous thromboembolism in cancer patients led to the use of anticoagulants including unfractionated heparin (UFH). Later trials showed that low molecular weight heparins (LMWH) were as effective as UFH [1]. When the results of several experimental studies were analyzed, the use of heparins in cancer patients appeared to provide a positive impact on the mortality beyond their antithrombotic effect; this was especially true for LMWHs compared to UHF [2].

The wide range of biological activities exerted by heparins result from their biochemical properties. Heparins are negatively charged polysaccharides with an ability to bind a variety of molecules and to influence their activity. The interactions between heparins and other molecules are mediated by certain physicochemical characteristics such as sequence composition, sulfation pattern, charge distribution, overall charge density, and molecular size [3]. The majority of the pro-angiogenic (e.g. fibroblast growth factors, vascular endothelial growth

factor, and tissue factor) and anti-angiogenic (e.g. tissue factor pathway inhibitor, endostatin, and angiostatin) endogenous substances can bind to heparin [2]. LMWHs are, on average, 5000 KDa weighing fragments of UFH obtained by enzymatic or chemical depolymerization under control [4].

Angiogenesis which is described as the formation of new blood vessels is a complex process including endothelial cell activation, controlled proteolytic degradation of the extracellular matrix, proliferation and migration of endothelial cells, and formation of capillary vessel lumina. It has been postulated that angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) can bind to and be stored in heparan sulfate proteoglycans present on the surface of endothelial cells and in the extracellular matrix. Soluble heparins compete with heparan sulfate to reverse this effect. Heparins induce increased levels of tissue factor (TF) pathway inhibitor in plasma and have been demonstrated to hinder TF production in stimulated human monocytes. In vitro experiments showed that heparins can influence angiogenesis by changing the structure of fibrin matrices [3]. The formation of capillary-like tubular structures after activation of microvascular endothelial cells is enhanced by UHF forming a more porous fibrin matrix and hindered by LMWHs forming a more rigid fibrin matrix [5]. To sum up, heparins may influence angiogenesis through modulation

Abbreviations: bFGF, basic fibroblast growth factor; LMWH, low molecular weight heparin; TF, tissue factor; UFH, unfractionated heparin; VEGF, vascular endothelial growth factor.

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of the expression of angiogenic growth factors and their inhibitors. The binding of these growth factors to their receptors seems to be improved by UHF and inhibited by LMWHs [3].

A number of *in vivo* assays have been proposed to study angiogenesis. These assays utilized various animal species including mammals (e.g. rats, mice), birds (e.g. chicken, quail), and fish (namely zebra fish). Chorioallantoic membrane (CAM) is a highly vascularized tissue of the avian embryo, specialized in respiration [6]. It is formed by the fusion of the (splanchnic) mesodermal layer of the allantois and the adjacent (somatic) mesodermal layer of the chorion. This double layered mesoderm has a very rich vascular network. The extracellular matrix of the CAM promotes angiogenesis through its content of fibronectin, laminin and collagen type IV, and the distribution of specific glycosaminoglycans. The CAM model has long been preferred for the studies on tumor angiogenesis and metastasis, as well as the studies of the macromolecules with angiogenic or antiangiogenic activity. After stimulation with an angiogenic compound, the vessel density increases whereas the vessels become less dense and eventually disappear with an antiangiogenic compound [7]. Because of its simplicity and low cost, CAM is the most commonly utilized *in vivo* angiogenesis model [2].

There are several forms of LMWH available for clinical practice. Nadroparin, enoxaparin, tinzaparin, and bemiparin are some of these forms. In the present study, we aimed to demonstrate and compare the antiangiogenic potency of these four types of LMWH in the chick embryo chorioallantoic membrane (CAM) model.

Materials and methods

Preparation of the pellets

In this trial, the effects of bemiparin sodium (Hibor® 3500 IU/0.2 ml, Laboratorios Farmaceuticos Rovi SA, Spain), enoxaparin sodium (Clexane® 8000 anti-Xa IU/0.8 ml, Aventis Intercontinental, France), nadroparin calcium (Fraxiparine® 1900 IU/0.2 ml, Glaxo Smith Kline, UK) and tinzaparin sodium (Innohep® 10000 IU/2 ml, Leo Pharma, Denmark) were studied. All low molecular weight heparins were in their commercially available form as soluble infusion. The agarose (Merck, Darmstadt, Germany) is added to distilled water to obtain a 2.5% (w/v) solution. This solution is put into the autoclave in 121 °C and under 1 atmospheric pressure to provide dissolution and sterilization. Subsequently, it is let to be cooled in a sterile container up to 37 °C. The study drug is added at this stage.

Appropriate volumes of solutions were used to achieve three different concentrations of the drugs (100 IU, 10 IU and 1 IU per 10 µl-pellet). For instance, to prepare the 10 µl-pellets containing 100 IU bemiparin, each pellet had to contain 5.71 µl of Hibor® and 4.29 µl of agarose solution. Approximately one hundred pellets for each study set are used. Therefore, approximately 1 ml of combined agar and drug solution (10 µl × 100 = 1 ml) was prepared initially for each drug. The drug solutions with 10 IU and 1 IU/10 µl concentrations were prepared by diluting these initial mixtures ten folds with the agarose solution again.

Using a micropipette, 10 µl drops of this mixed solution were placed on previously sterilized, vertical, cylindrical stainless steel rods which were 5 mm in diameter to obtain circular pellets with the same diameter. The pellets were then let to be solidified at room temperature in a sterile setting.

Chicken chorioallantoic membrane (CAM) assay

Ross 308 strain fertilized hens' eggs were obtained from Yemsel Poultry Company (Kayseri, Turkey). The study protocol was approved by the Cumhuriyet University Animal Ethics Committee.

The fertilized hens' eggs were incubated in horizontal position with environmental conditions of 37.5 °C temperature and 80%

relative humidity. On the fifth day of the incubation period, 5 ml of albumen was taken through the eggshell with a syringe (Fig. 1A) and a shell piece of 2–3 cm in diameter was removed from the contrary side of the eggs. Normal development of the CAM was verified (Fig. 1B) and malformed or dead embryos were excluded. The windows on the egg shells were sealed with gelatin and thereafter, the eggs were incubated for 72 more hours to have CAM reaching 2 cm in diameter. Subsequently (on day 8), the seal was removed and the pellets were placed on the chorioallantoic membrane of each egg (Fig. 1C). The seal was placed again and the eggs were then incubated for 24 hours. The angiogenesis level was evaluated after that period.

For each tested drug solution, twenty eggs were used. As the control group, pellets containing just agar were utilized. All the tests were duplicated. The eggs in which the pellets caused inflammation and embryo toxicity were excluded.

Angiogenesis scoring

The inhibitory effects of the drugs on angiogenesis in chorioallantoic membrane were evaluated under a stereoscopic microscope and assessed according to the scoring system used previously in several studies [8,9]. In this scoring system, the change in the density of the capillaries around the pellet and the extent of the effect are evaluated (Fig. 1D). In the initial scoring of each subject, score 0 indicated the absence of any demonstrable antiangiogenic effect (normal embryo and no difference in surrounding capillaries); score 0.5 represented a very weak (no capillary-free area but an area with reduced density of capillaries which is not larger than the pellet area), score 1 a weak-moderate (a small capillary-free area or a small area with significantly decreased density of capillaries; less than double the size of the pellet is involved), and score 2 a strong antiangiogenic effect (a capillary-free area around the pellet which is equal to or more than double the size of the pellet itself). The equation used for the determination of the average score was as follows:

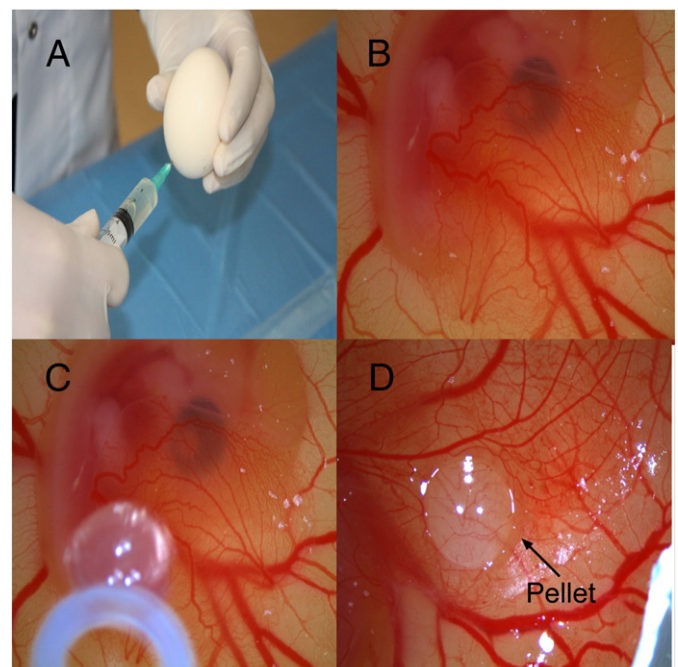
$$\text{Average score} = \frac{[\text{Number of eggs (Score 2)} \times 2 + \text{Egg number (Score 1)} \times 1]}{[\text{Total number of eggs (Score 0, 1, 2)}]}$$


Fig. 1. A. Albumen is removed with a syringe. B. The appearance of the chorioallantoic membrane (CAM) through a window on the egg shell (×8). C. The placement of the pellet on the CAM (×8). D. Inhibition of the capillaries on the CAM (score: 0.5) by the drug (×8).

According to this scoring system, a score of <0.5 meant that there was no antiangiogenic effect; a score of 0.5 to 1 indicated a weak antiangiogenic effect, and a score of >1 implied a strong antiangiogenic effect.

Statistical analysis

The scores of angiogenesis were compared with Kruskal-Wallis ANOVA test and Mann-Whitney *U* test. A *p* value of less than 0.05 was considered as statistically significant.

Results

The eggs on which a 10 μ l-agarose pellet with no drug was installed demonstrated no significant antiangiogenic effect (average antiangiogenic score = 0.2). All the study drugs demonstrated some antiangiogenic effect compared to the negative control ($p < 0.05$). Each tested drug is evaluated separately and the results with different solutions were compared.

Fig. 2 shows the antiangiogenic scores of bemiparin (A), enoxaparin (B), nadroparin (C), and tinzaparin (D) in 100 IU, 10 IU, and 1 IU/10 μ l concentrations. As shown in the scatter graph, the antiangiogenic score with the 100 IU-solution of bemiparin was significantly higher than those with 10 IU- and 1 IU-solutions ($p = 0.034$). The antiangiogenic score of the enoxaparin solution with 100 IU of drug was significantly higher than those with 10 IU and 1 IU of drug ($p = 0.041$).

The antiangiogenic score with the 100 IU-solution of nadroparin was significantly higher than those with 10 IU- and 1 IU-solutions ($p = 0.029$). Furthermore, the antiangiogenic score of the nadroparin solution with 10 IU of drug was significantly increased compared to that with 1 IU of drug ($p = 0.013$). The antiangiogenic scores of tinzaparin solution with 100 IU of drug was significantly higher in comparison to those with 10 IU and 1 IU of drug ($p = 0.044$). Moreover, the

antiangiogenic score with the 10 IU-solution of tinzaparin was significantly higher than that of the 1 IU-solution ($p = 0.022$).

Discussion

Angiogenesis can be described as the new vessel formation if required. This formation is controlled by the factors released from the surrounding tissues. It has been stated that, low-molecular-weight heparin (LMWH) could demonstrate its effectiveness by inhibition of binding of angiogenic growth factors such as basic fibroblast growth factor (bFGF) and VEGF to their receptors, by inhibition of expression of the TF factor, and by affecting the fibrin structure. Heparin fragments with less than 18 saccharides hinder VEGF activity and those with less than 10 saccharides inhibit bFGF activity. Compared to UHF, small molecular heparin fragments are more efficacious in inhibiting VEGF- and bFGF-mediated angiogenesis in vivo [10].

Various methods are used to produce LMWH from heparin, including nitrous acid depolymerization for nadroparin and dalteparin, benzylation followed by alkaline depolymerization for enoxaparin and bemiparin, peroxidative depolymerization for ardeparin, and enzymatic depolymerization with heparinase for tinzaparin. Because LMWHs are generated with different ways of depolymerization, their pharmacokinetic features may vary and they may not be clinically interchangeable [4]. LMWHs may also differ in their antiangiogenic potentials due to several factors which may include the mean fragment size, the manufacturing process, the assay used to assess the effect, and the type of angiogenesis reaction studied [2]. LMWHs have a mean molecular weight of 5000 Da. There is a linear relationship between the number of saccharide units and molecular weight (e.g. 12 units weigh 3600 Da, 18 units weigh 5400 Da) [4]. Bemiparin has a mean molecular weight of 3600 Da, enoxaparin 4200 Da, nadroparin 4500 Da, and tinzaparin 4500 Da [11]. LMWHs are postulated to exert their effect through suppression of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor

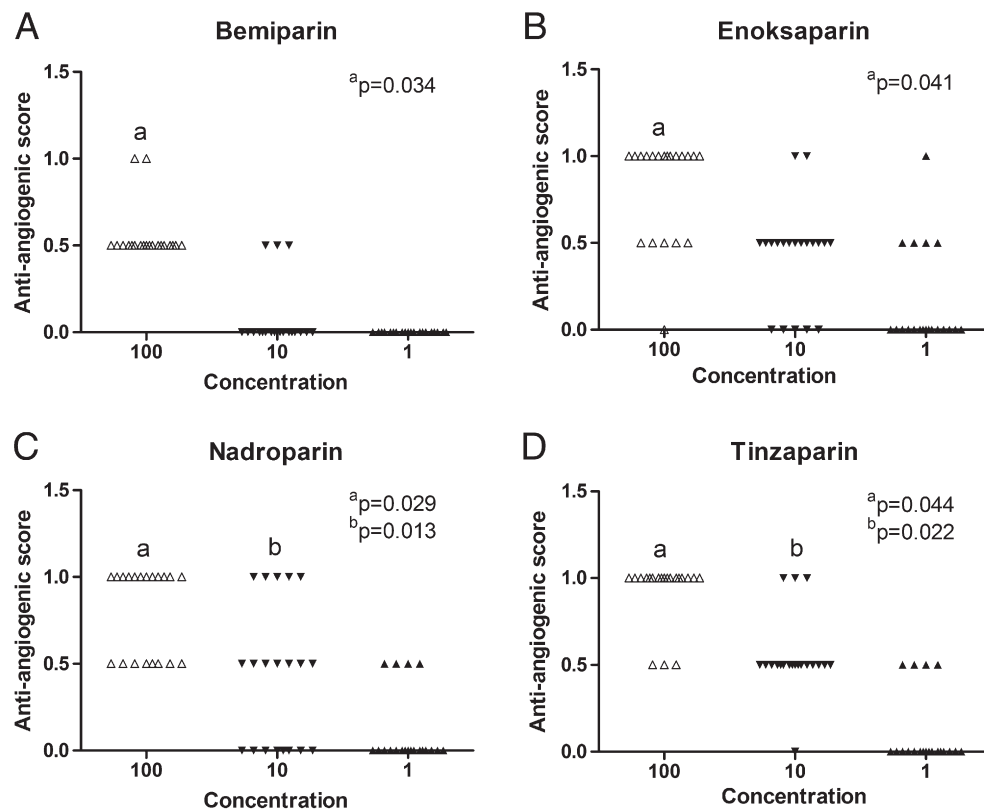


Fig. 2. The antiangiogenic scores of bemiparin (A), enoxaparin (B), nadroparin (C), and tinzaparin (D) in 100 IU, 10 IU, and 1 IU/10 μ l concentrations.

(bFGF)-induced angiogenesis [2]. Heparin fragments containing less than 18 oligosaccharide units (weighing less than 5.4 kDa) inhibit the binding of VEGF on its receptors on endothelial cells in vitro [2].

Previous studies using in vivo models examined the effects of individual LMWHs, sometimes in comparison to UFH. In a study with chick embryo CAM model conducted by Mousa and Mohamed, tinzaparin has been demonstrated to have a dose-dependent antiangiogenesis effect [12]. Nasir et al. observed in heparin knockout mice the antiangiogenic effect of the dalteparin on an experimental tumour model [13]. Fernandez et al. compared the antiangiogenic effects of UFH and dalteparin using in vitro, ex vivo (chick aortic ring model) and in vivo angiogenesis models [14]. On the other hand, Norrby and Nordenhem demonstrated uniquely angiogenic effect of a LMWH, namely dalteparin, in a rat mesenteric assay [15]. According to our results with tinzaparin, nadroparin, enoxaparin, and bemiparin, these LMWHs have obvious antiangiogenic effects as shown in CAM model which is a convenient angiogenesis study model. This study allowed us to categorize the four different LMWHs in respect to the potency of their effects on angiogenesis. We utilized a wide range of concentrations of 100 IU, 10 IU and 1 IU per 10 μ l agarose solution. The choice of these concentrations had to be somewhat arbitrary because of the lack of previous studies standardizing these values.

This is, as far as we know, the only study using CAM model to investigate the impact of multiple LMWHs in the same setting. Our results suggest that, being more significant in high concentrations (100 IU/10 μ l), bemiparin sodium, enoxaparin sodium, nadroparin calcium and tinzaparin sodium have antiangiogenic effects on chick chorioallantoic membrane. The fact that nadroparin calcium and tinzaparin sodium have also substantial antiangiogenic effects at the moderate concentration of 10 IU/10 μ l may imply their higher antiangiogenic potency. A possible categorization of LMWHs in this context would ease the choice of drug that would be used in further experimental and clinical research.

Conflict of interest statement

The authors declare that they have no conflict of interest regarding the content of this article. No funding was received for this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.thromres.2011.05.005.

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