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The Effect of Low Molecular Weight Heparins on Placentation: A Rat Model Study

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ABSTRACT

Low molecular weight heparins (LMWHs) have been used for the treatment for recurrent pregnancy loss (RPL) for a long time. We aimed to investigate the efficacy of the LMWHs on angiogenesis and apoptosis during placentation. A total of twenty-four rats were randomly divided into three groups each containing 8 rats: normal saline; enoxaparine sodium 0.4 ml, and enoxaparine sodium 0.8 ml were given to the Group 1, 2 and 3, respectively. Normal saline and enoxaparine sodium 0.4 ml or 0.8 ml were given to the rats beginning on the day the pregnancy was detected and continued until the 15th day of the pregnancy. The tissues containing placental decidual zone were immunostained for vascular endothelial growth factor A (VEGF-A) and caspase 7. The decidual and placental VEGF-A and the decidual caspase 7 immunostaining scores of all of the groups were high, however, there were no statistically significant differences among the groups ($p>0.05$). On the other hand, the placental caspase 7 immunostaining scores of the normal saline group were significantly lower than those of the enoxaparine sodium 0.4 and the enoxaparine sodium 0.8 groups ($p<0.05$). LMWHs seem to have effects on placental angiogenesis and apoptosis.

Keywords: Low molecular weight heparin, Placenta, Angiogenesis, Apoptosis.

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Introduction

Recurrent pregnancy loss (RPL) is an important obstetric health issue. Although there are several etiological factors for RPL, no identifiable underlying cause could be found in approximately half of the couples [1]. Certain hereditary thrombophilias, which are responsible for half of the thromboembolic conditions occurred during pregnancy, thought to be the cause in the idiopathic RPL cases [2, 3]. In these cases, thrombogenic predisposition during pregnancy further conributes to hypercoagulability and may cause uteroplacental blood flow decline, placental thrombosis and pregnancy loss, because placental perfusion should be sufficient for a healthy pregnancy [4, 5].

The appropriate formation and maturation of vascular bed is of paramount importance for healthy placental development [6]. Two basic mechanisms responsible for the development of placental vascular bed are vasculogenesis and angiogenesis [7]. Also, apoptosis and cell proliferation should be appropriately balanced for placentation and remodelling during pregnancy [8]. Therefore, apoptosis related changes in the embrionic and the extra-embrionic tissues may cause congenital structural abnormalities and pregnancy loss [9]. It has been recently shown that trophoblast invasion abnormalities occurred during early pregnancy may cause pregnancy loss [10]. The management of thrombophilic asymptomatic pregnant women is controversial and ampiric. Antithrombotic treatment before conception and/or during early pregnancy is usually recommended [11]. The most widely used antithrombotics during pregnancy are the unfractioned heparin (UFH) and the low molecular weight heparins (LMWHs) [12]. LMWHs have replaced UFH and have been widely used for years because of their ease of use and safety profile [13]. Enoxaparine sodium is one of the most preferred LMWHs because of its safety profile, tolerability and availability [14].

In this study, we aimed to investigate the effect of LMWHs on angiogenesis and apoptosis during placentation to clarify the mechanisms of the efficacy of the LMWHs on live birth rates in RPL cases.

Materials and Methods

Materials

The rats used in the experiments were obtained from Cumhuriyet University Experimental Animal Unit. Four months-old, 200-220 gram weighted Wistar albino female rats were used in the experiments. Clexane sterile solution (Aventis Pharma, İstanbul, Türkiye) containing 100 mg enoxaparine sodium, equivalent to 10,000 anti-Xa IU was given to the rats in either 0.4 ml or 0.8 ml doses. Immunostaining was performed by using Ventana Benchmark XT automated slide-staining system (BenchMark XT Staining Module, Ventana Medical Systems) for VEGF-A antibodies (Rabbit Polyclonal Antibody Thermo Scientific, US) and caspase 7 antibodies (Rabbit Polyclonal Antibody Thermo Scientific, US).

Methods

This study was approved by Cumhuriyet University Faculty of Medicine, Animal Trials Ethics Committee (01.15.2015, approval number: 6). For conception, adult male rats were placed into the cages during 05:00 pm and 09:00 am for five consecutive days (throughout a menstrual cycle). Every next day, vaginal examination with a pediatric otoscope (HEINE mini 2000, Heine Optotechnik, Herrsching, Germany) was performed to detect copulatory plug, which is an indicator of mating. The day copulatory plug detected was considered as the first day of the pregnancy. Twenty-four rats were randomly divided into the three groups each containing 8 rats: normal saline, enoxaparine sodium 0.4 ml, and enoxaparine sodium 0.8 ml were given to the Group 1, 2 and 3, respectively. 1 mg (0.01 ml) enoxaparine sodium is equivalent to 100 anti-Xa IU. Human equivalent doses were given to the rats. The rats were given 1 mg/kg body weight enoxaparine sodium subcutaneously.

The groups were treated as follows:

1. Normal saline group: 0.1 ml/day saline, subcutaneously
2. Enoxaparin sodium 0.4 group: 0.1 ml/day enoxaparine sodium (Clexane, Aventis Pharma, İstanbul, Türkiye), subcutaneously
3. Enoxaparin sodium 0.8 group: 0.1 ml/day enoxaparine sodium (Clexane, Aventis Pharma, İstanbul, Türkiye), subcutaneously

On the 15th day of the pregnancy, the rats were anesthetized with intramuscular ketamine 90 mg/kg body weight and Xylazine 3 mg/kg body weight. After cervical dislocation, rats underwent a laparotomy and their uteri containing pregnancy material were removed.

Histopathology

Placental tissues were carefully separated from embryos and cuts containing placenta-decidual zone were obtained. The tissues were then stained with haematoxylin and eosin and examined with light microscopy by a pathologist (H. Ö.) blinded for the groups.

Immunohistochemistry

For immunohistochemistry analyses, tissue sections at 3 µm thickness were obtained from paraffin-embedded blocks containing placental-decidual zone and transferred onto positively charged surface. Renal tissue for VEGF-A and duodenal tissue for caspase 7 were used as positive controls. Cytoplasmic staining pattern was considered positive for VEGF-A and caspase 7. Immunostaining was scored with regard to quantity and intensity of positively stained cells as follows: for intensity of staining: negative (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining); for quantity of staining: the percentage of stained areas: <10%, 1; 10-50%, 2; 51-80%, 3; and >80%, 4. The final immunostaining score was obtained by multiplying the quantity score and the intensity score. Scores 0-1 was considered as negative or low; 2-6 as moderate; and 8-12 as high expression. Statistical analyses were performed for each antibody used.

Statistical Analysis

A statistical software package program (SPSS, ver. 14.0) was used for statistical analyses. Kruskal Wallis test, Mann Whitney U test and Chi-Square test were used as approprite. A p value of <0.05 was considered statistically significant.

Results

The mean (±SD) decidual VEGF-A staining scores were 8.75 ± 2.12, 9.62 ± 1.99 and 10.62 ± 1.92 in the normal saline, enoxaparine sodium 0.4 and the enoxaparine sodium 0.8 groups, respectively. The mean (±SD) placental VEGF-A staining scores were 10.75 ± 2.37, 11.50 ± 1.41 and 12.00 ± 0.00 in the normal saline, enoxaparine sodium 0.4 and the enoxaparine sodium 0.8 groups, respectively. There were no statistically significant differences among the groups regarding the decidual and the placental VEGF-A staining scores (p>0.05, Table 1).

Table 1: Decidual and Placental VEGF-A Staining Scores

	Normal saline (n=8)	Enoxaparine sodium 0.4 (n=8)	Enoxaparine sodium 0.8 (n=8)	Results
VEGF-A Decidua				
Mean ± SD	8.75 ± 2.12	9.62 ± 1.99	10.62 ± 1.92	KW=3.75
Median	8.00	8.50	12.00	P=0.153
VEGF-A Placenta				
Mean ± SD	10.75± 2.37	11.50± 1.41	12.00 ± 0.00	KW=2.27
Median	12.00	12.00	12.00	P=0.320

SD=standard deviation

The mean (±SD) decidual caspase 7 staining scores were 11.00 ± 1.85, 11.12 ± 1.64 and 11.25 ± 1.38 in the normal saline, enoxaparine sodium 0.4 and the enoxaparine sodium 0.8 groups, respectively. There were no statistically significant differences among the groups regarding the decidual caspase 7 staining scores (p>0.05, Table 2)

Table 2: Decidual and Placental Caspase 7 Staining Scores

	Normal saline (n=8)	Enoxaparine sodium 0.4 (n=8)	Enoxaparine sodium 0.8 (n=8)	Result
Caspase 7 Decidua				
Mean ± SD	11.00 ± 1.85	11.12 ± 1.64	11.25 ± 1.38	KW=0.07
Median	12.00	12.00	12.00	P=0.096
Caspase 7 Placenta				
Mean ± SD	8.50 ± 0.53	9.37 ± 1.06	9.37 ± 1.06	KW=8.46
Median	8.50	9.00	9.00	P=0.0014*

SD=standard deviation, *p<0,05 statistically significant

The mean (±SD) placental caspase 7 staining scores were 8.50 ± 0.53, 9.37 ± 1.06 and 9.37 ± 1.06 in the normal

saline, enoxaparine sodium 0.4 and the enoxaparine sodium 0.8 groups, respectively. The difference among the groups was found statistically significant, the normal saline group had lower placental caspase 7 staining scores than those of the enoxaparine sodium groups, whereas the enoxaparine sodium 0.4 and the enoxaparine sodium 0.8 groups did not differ from each other ($p<0.05$ for normal saline vs. enoxaparine sodium 0.4 and normal saline vs. enoxaparine sodium 0.8; $p>0.05$ for enoxaparine sodium 0.4 and enoxaparine sodium 0.8) (Table 2).

Light microscopy examination of H-E stained tissues revealed that chorioamnionitis were present in the enoxaparine sodium groups and widespread perivillous fibrine deposition was present in the normal saline group.

Discussion

In this study, we aimed to investigate the effect of LMWHs on angiogenesis and apoptosis during placentation and thus understand their reported efficacy of increasing live birth rates in patients with RPL, an obstetric condition with controversial issues regarding its etiology and management. We preferred to use enoxaparine sodium in our experiment because of its safety, tolerability and availability [14]. The reason we used rats in our experiment is that human and rat placenta have many structural and developmental similarities such as hemochorial placentation and rat placenta model is widely used in placental developmental studies [15].

It is known that disorders and deficiencies in placental development cause pregnancy complications [16]. For ethical reasons, it is not possible to carry out studies on the human placenta to understand the potential underlying mechanisms. For this reason, although there are limitations, placenta studies are carried out on animal models. Correct placental development is essential for embryonal and fetal development. The placenta is composed of trophoblast and endothelial cells, and it is a structure where complex molecular interactions of maternal and fetal factors occur. It also provides nutrient and gas exchange between mother and baby, helps the adaptation of the mother's body to pregnancy and acts as a protective barrier for the fetus [17]. The outer layer of the placenta consists of two parts, the inner layer called cytotrophoblast and the outer layer called syncytiotrophoblast. In a normal placental development, cytotrophoblasts settle in the uterus and invade the spiral arteries from the uterine wall. Spiral arteries lose their musculoelastic layer as a result of invasion of cytotrophoblasts, and peripheral resistance and blood pressure decrease in spiral arteries, resulting in the physiological properties required for adequate perfusion of the placenta [18, 19].

Angiogenesis is a vital process in placental development and therefore in embryonal and fetal life. A balance of pro- and anti-angiogenic factors is essential for successful placentation. VEGF has a critical role in the maintenance of blood vessel endothelium and structure as well as in angiogenesis [20].

Pregnancy related thrombogenic changes contribute to underlying hypercoagulopathy in patients with RPL and cause uteroplacental blood flow decline, placental thrombosis and pregnancy loss [4]. Sufficient placental perfusion is fundamental for a healthy pregnancy [5]. Multiple factors controlling vasculogenesis, angiogenesis and trophoblast functions play role in the process of placentation [21].

Antithrombotic treatment before conception or early pregnancy is recommended in women with thrombophilia [11]. The antithrombotic medications of choice during pregnancy are UFH and LMWHs. Currently, LMWHs are the antithrombotic medications most widely used [12]. It has been suggested that LMWHs also have effects on placental function and invasion in addition to their anticoagulant and antiinflammatory properties [22-24]. Thrombosis and infarctions causing placental insufficiency have been reported in patients with thrombophilia and unfavorable obstetric history [25]. Sarto et al. [26] have reported that LMWHs increased live birth rates from 15% to 85% in patients with hereditary thrombophilia and RPL.

UFH and LMWHs have been reported to promote angiogenesis in in vitro healthy first and second trimester placental material [27]. In our study we found that the decidual and the placental VEGF-A scores of the enoxaparine sodium groups were higher than those of the saline group and these scores of the enoxaparine sodium 0.8 group were also higher than those of the enoxaparine sodium 0.4 group, however, these differences did not reach statistical signifance. These findings may be due to small sample size in our study. Further studies are warranted.

The presence of apoptotic cells in placental tissue is controversial. Apoptotic cells have been reported in the rat endometrial stromal cells at the fifth day of the pregnancy [28]. On the other hand, Perez et al. [29] have reported the absence of apoptotic cells in the normal placental tissue at the 14th day of the pregnancy. We have found increased apoptosis in the placental tissue at the 15th day of the pregnancy in all of the experimental groups including the normal saline group. However, placental caspase 7 immunostaining scores were higher in enoxoparine groups than those of the normal saline group. Our findings indicate that apoptosis is present in rat placental tissue at the 15th day of the pregnancy and enoxaparine sodium may increase the apoptotic activity. LMWHs have been reported to decrease apoptotis via Bcl-2 activation and Bax supression in rat placenta [30]. These findings are contradictory to ours. More information is needed to clarify the effect of LMWHs on apoptosis in placental tissue. LMWHs may act differently in pathological conditions suc as RPL.

Fibrine accumulation surrounding villi is a frequent finding, it can be seen in one fourth of uncomplicated term pregnancies. However, true massive perivillous fibrine accumulation involving 80-90% of the villous parenchyma can cause fetal death [31]. Diffuse perivillous and intervillous fibrine accumulation possibly caused by perfusion problems in the terminal villi, have been

reported in preeclampsia patients [32, 33]. In our study, only normal saline group had diffuse perivillous fibrine accumulation. This findings indicate that LMWHs can have beneficial effects on placental perfusion.

In conclusion, LMWHs seem to have effects on placental angiogenesis and apoptosis. The small sample size of our study may preclude to detect statistically significance. Further studies with larger sample sizes and molecular analyses are warranted.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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