Investigation of the effects of heparin and low molecular weight heparin on E-cadherin and laminin expression in rat pregnancy by immunohistochemistry

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BACKGROUND: Heparin and low molecular weight heparin (LMWH) are used widely to improve the pregnancy outcome in women with thrombophilia, miscarriage, recurrent miscarriage and fetal death. This study was designed to investigate the effects of heparin and LMWHs, enoxaparin and tinzaparin, on E-cadherin and laminin expression in placental and decidual tissues in rat pregnancy. METHODS: Wistar albino female rats (n = 48) were randomly assigned to four study groups (normal saline, heparin, enoxaparin and tinzaparin) in the preconceptional period. Tissue sections of placenta and decidua were immunohistochemically examined for the expression of E-cadherin and laminin. RESULTS: E-cadherin placental staining score of heparin group was significantly lower and E-cadherin decidual staining score of heparin and enoxaparin groups were significantly lower than control group. There were no significant differences in placental and decidual laminin staining scores among the study groups. CONCLUSIONS: Heparin and enoxaparin can reduce E-cadherin expression but not laminin expression in rat pregnancy. They might modulate trophoblast invasion. We suggest that this is the possible underlying mechanism involving in improvement of trophoblast invasion by the use of heparin and LMWH in patients with the history of miscarriage.

Key words: e-cadherin/enoxaparin/laminin/pregnancy/tinzaparin

Introduction
Heparin and its derivative low molecular weight heparin (LMWH) are used widely to improve the pregnancy outcome in women who have thrombophilia and previous pregnancies that have been complicated by miscarriage, recurrent miscarriage and fetal death (Alikani, 2005; Christiansen et al., 2005). The use of heparin to prevent pregnancy loss was based on the premise that some pregnancy losses were caused by placental thrombosis and infarction and that thromboprophylaxis with heparin could prevent this process; however, recent data suggested that pregnancy losses might also be associated with defective decidual endovascular trophoblast invasion (Kutteh, 1996). Successful pregnancies have also been achieved with LMWH in women with recurrent miscarriage of unknown aetiology (Miyashita et al., 2003). It seems likely that different mechanisms are involved in the effects of heparin and LMWH in the treatment of pregnancy loss with unknown aetiology.

Among the many types of adhesion receptors on the cell surface, cadherins, integrins, the immunoglobulin superfamily and selectins participate in mediating vital biological events such as embryogenesis, cell growth and differentiation (Frenette and Wagner, 1996). Cadherins are a group of cell adhesion proteins that mediate Ca2+-dependent cell–cell adhesion, a fundamental process required for embryonal development and blastocyst implantation (Frenette and Wagner, 1996; Floridon et al., 2000). The adhesion properties led to the hypothesis that various cadherins might mediate specific cell–cell adhesion and play a pivotal role in the formation and maintenance of tissues (Suzuki, 1996). A large number of cadherins and cadherin-related proteins are expressed in different tissues of a variety of multicellular organisms. In human placenta, E-cadherin is involved in trophoblast differentiation and invasiveness (Xue et al., 2003).

In multicellular organisms, interactions between the cells and the extracellular matrix (ECM) are essential for the morphogenesis and maintenance of all organs and tissues (Kedinger et al., 2000). Basement membrane (BM) is formed by a complex set of collagenous and noncollagenous glycoproteins such as laminins, nidogen and proteoglycans, and has a unique composition in each organ (Yurchenco et al., 2004). Laminins are a family of multifunctional macromolecules, ubiquitous in BMs, and represent the most abundant structural noncollagenous glycoproteins of these highly specialized ECMs. Laminins therefore have a central role in the formation, the architecture
and the stability of BMs. In addition, laminins may both separate and connect different tissues, that is the parenchymal and the interstitial connective tissues. They also trigger and control cellular functions. Trophoblast cells maintain strong cell–cell contacts on substrates of laminins and exhibit strong staining of VE-cadherin in all regions of cell–cell contact. Laminin isoforms influence the direction and quality of invasion of trophoblast cells during implantation (Klaffky et al., 2006). The consequent phenotypes highlight the pivotal role of laminins in determining heterogeneity in BM functions (Aumailley and Smyth, 1998).

Because LMWH is used increasingly often during pregnancy, investigations of the possible underlying mechanisms of their effects seem to be required, but few studies have examined their effects in the first trimester specifically. The aim of this study was to evaluate the effects of heparin and LMWHs, enoxaparin and tinzaparin, on E-cadherin and laminin expression in the placental and decidual tissues in rat pregnancy.

Materials and methods

Animals

All procedures were approved and performed under the guidelines of the Animal Ethics Committee of Cumhuriyet University School of Medicine. Experiments were carried out on Wistar albino female rats (190–285 g, body weight) maintained in individual cages under standard laboratory conditions. Rats were fed with standard chow diet and water ad libitum and allowed 1 week of adjustment to their new environment. Each rat was assigned an identification pairing number before mating. Attempts at conception were undertaken for up to 5 consecutive days (one estrous cycle), and rats were mated between 17:00 and 09:00 h. The next day, vaginal examination was performed to visualize the copulatory plug, a waxy congealed plug in the vagina of the female, as an indication of mating with a paediatric otoscope (Heine mini 2000, Heine Optotechnik, Herrsching, Germany). We have begun to use the study drugs after the first vaginal plug detection. If the rats have not yet become pregnant, we repeated their mating until the rats got pregnant. The day of the last discovery of the vaginal plug (09:00 to 11:00 h) was counted as embryonic day 0. The rats ($n = 48$) were randomly assigned to four study groups on gestational day 0: Normal saline ($n = 12$), heparin ($n = 12$), enoxaparin ($n = 12$) and tinzaparin ($n = 12$).

Treatment protocol

In all the study groups, following treatments were administered on gestational day 0. In control group, rats were treated with normal saline 0.3 ml/day s.c. In heparin group, rats were treated with 16 IU/0.3 ml s.c. daily (Liquemine® 25.000 IU/5 ml, Roche, Istanbul, Turkey). In enoxaparin group, rats were treated with enoxaparin 0.3 mg/0.30 ml s.c. daily (Clexane®, enoxaparin sodium, 0.4 mg, Aventis, Istanbul, Turkey). In tinzaparin group, rats were treated with tinzaparin 35 anti-Xa IU/0.3 ml subcutaneously daily (Innohep®, tinzaparin sodium 9.000 anti-Xa IU, 0.9 ml, Abdi Ibrahim, Istanbul, Turkey). The rats were killed by cervical dislocation on gestational day 15 and then median laparotomy was performed, and uterine horns including pregnancy material were excised and stored in 10% formaldehyde.

Histopathological examination

Embryos in each uterine horn were excised, and conjunction site of placental-uterine tissues were routinely fixed in 10% buffered formalin (pH = 7.2) and embedded in paraffin wax. Tissue sections (5 μm in thickness) were cut from paraffin-embedded blocks, mounted on polylysine-coated glass slides and dried in an oven overnight at 37°C. The sections were dewaxed in xylene and rehydrated through graded concentrations of alcohol. Endogenous peroxidase activity was blocked using 3% H2O2 in methanol. Immunohistochemistry was performed using the avidin–biotin complex peroxidase assay. Antibodies for E-cadherin: mouse monoclonal antibody, cadherin-E/E-cadherin Ab-3, ready-to-use (Clone 36B5) (Lab Vision Corp., Fremont, CA, USA) and laminin (rabbit polyclonal antibody laminin Ab-1, ready-to-use, Lab Vision Corp.) were applied, and the sections incubated at room temperature for 30 min biotinylated goat anti-mouse/rabbit antibody was used as the linker molecule and amino ethyl carbazole as the chromogen. A light haematoxylin counterstain was used. Sections from normal colon mucosal epithelium and renal tissue were used as positive controls for E-cadherin and laminin antibodies, respectively, and replacement of the primary antibody with Tris-buffered saline was used as negative control. Evaluation of the staining was carried out by two pathologists (S.A. and A.M) without referring to the clinical or histopathological features and scored according to the staining intensities of E-cadherin and laminin by an independent assessment simultaneously. For E-cadherin, scores 1–4 were allocated as follows: 1, negative; 2, weak staining; 3, moderate staining and 4, intense staining (Figure 1) (Balaram et al., 2004). For laminin, scores 1–3 were allocated as follows: 1, negative; 2, patchy staining; 3, linear staining (Figure 2) (Korhonen and Virtanen, 2001). Linear staining as a histopathologic definition is used for continuous staining of the basement membrane.

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Figure 1. Placental and decidual E-cadherin staining. (A) Strong E-cadherin staining in placenta in control group (original magnification ×50). (B) Strong E-cadherin staining in decidua in control group (original magnification ×50). (C) Mild staining in decidua and placenta in heparin group (original magnification ×25). (D) Strong E-cadherin staining in placenta and mild staining in decidua in enoxaparin group (original magnification ×25).

Figure 2. Laminin staining in placenta and decidua. (A) Continuous linear staining in placenta and decidua in control group (original magnification ×50). (B) Representative section showing linear and patchy staining in placenta and decidua in heparin, enoxaparin and tinzaparin groups (original magnification ×25).
Statistical analysis

Data were presented as mean ± SD. Pre-gestational and gestational day 15 weights, litter size and treatment period were analysed by one-way analysis of variance (ANOVA) with Student–Newman–Keuls multiple comparisons test as a post hoc test. Placental and decidual E-cadherin staining scores, placental and decidual laminin staining scores were analysed by Kruskal–Wallis ANOVA with Student–Newman–Keuls multiple comparisons test. A value of $P < 0.05$ was considered as statistically significant.

Results

Table I summarizes pre-gestational and gestational day 15 weights, litter size and treatment period of the study groups as mean ± SD. There were no significant differences in pre-gestational and gestational day 15 weights, litter size and treatment period among the study groups ($P > 0.05$). Figure 3 summarizes median E-cadherin placental staining score and median E-cadherin decidual staining score. Median E-cadherin placental staining score of heparin group [median (min–max)] was significantly lower than those of control, enoxaparin and tinzaparin groups [2(1–3) versus 3(2–4), 3(2–4) and 3.5(2–4), respectively] ($P < 0.05$). Median E-cadherin decidual staining score of heparin and enoxaparin groups were significantly lower than control group [1(1–3) and 2(1–3) versus 2.5(2–4), respectively] ($P < 0.05$). Figure 4 shows laminin placental and decidual staining scores. There were no significant differences in placental and decidual laminin staining scores among the study groups ($P > 0.05$).

Discussion

Herein, we investigated the E-cadherin and laminin expressions with immunohistochemistry in placental and decidual tissues of pregnant rats which received heparin, enoxaparin or tinzaparin throughout their pregnancies. We found that heparin reduced E-cadherin expression in placental tissue but not enoxaparin and tinzaparin. In decidual tissue, heparin and enoxaparin reduced E-cadherin expression but not tinzaparin. Heparin, enoxaparin and tinzaparin, however, did not cause any meaningful changes in laminin expression in both placental and decidual tissues. Several studies have been conducted on E-cadherin and its role in trophoblast invasion and confirm our findings. Expression of E-cadherin was decreased in gestational trophoblastic disease when compared with that in normal first trimester placenta (Xue et al., 2003). Floridon et al. (2000) also reported a temporary shift in E-cadherin expression in extravillous trophoblasts possessing a migrating and invasive potential. One of the reasons why heparin but not LMWH decreased E-cadherin expression in placental and decidual tissues is the effect of heparin on growth factors, including placental growth factor, heparin-binding epidermal growth factor and vascular endothelial growth factor (Chobotova et al., 2002). Shih et al. (2002), in their study, demonstrate that expression of E-cadherin in a E-cadherin negative human implantation site intermediate trophoblastic cell line (IST-1) results in a contact-mediated inhibition of motility and invasion and suggest an important role for E-cadherin down-regulation in the intermediate trophoblast during implantation. And also unfractionated heparin at therapeutic doses and LMWH at supratherapeutic doses promoted extravillous trophoblast differentiation, and both unfractionated heparin and LMWH inhibited hepatocyte growth factor-stimulated extravillous trophoblast motility at supratherapeutic doses (Quenby et al., 2004). It is suggested that the effects of heparin and LMWH on trophoblast invasion and motility might be one of the factors contributing to the mechanism in pregnancy loss. Defective decidual endovascular trophoblast invasion is the most frequent histological abnormality in early pregnancy loss and in pre-eclampsia, which is the leading cause of maternal and fetal mortality and morbidity (Quenby et al., 2004; van den Brule et al., 2005). However, the mechanism of recurrent miscarriage remains the subject of research (Di Simone et al., 1999). Immunohistochemical findings suggest that invasiveness of trophoblastic cells may in part be due to the loss of their adhesive properties mediated by E-cadherin (Shih et al., 2002).

According to Xue et al. (2003), abnormal expression of E-cadherin contributes abnormal invasive ability to trophoblasts and plays a role in the pathogenesis and progression of gestational trophoblastic diseases. Immunoreactivity of E-cadherin was reduced in choriocarcinoma and complete hydatidiform mole when compared with that in normal first trimester placenta. Molecular analysis of E-cadherin has recently been investigated in several human malignancies, including breast cancer, prostate cancer and Wilms’ tumour (Mutoh et al., 1993; Murray et al., 1995; Nelson-Piercy et al., 1997; Bankfalvi et al., 1999). Loss of E-cadherin expression has been correlated with the neoplastic transformation of epithelial cells (Nishiyama et al., 1997; Norman et al., 2002). Kokenyesi et al. (2003) have also found evidence to indicate that expression of the cell–cell adhesion molecule, E-cadherin, was inversely correlated with invasive phenotype of peritoneal carcinoma and ovarian carcinoma. Li et al. (2003), in their study, demonstrated that the expression of both E-cadherin and β-catenin showed a decreasing trend from first to third trimesters indicating an increased invasion potential. In pre-eclampsia, they have found an up-regulation of E-cadherin and β-catenin expression. In placenta accreta, the level of expression of both did not

| Table I. Maternal weights (pre-gestational and gestational day 15), litter size and treatment period of the study groups (mean ± SD) |
|-----------------------------------------------|-------------------|-------------------|-------------------|-------------------|
|                                              | Control           | Heparin           | Enoxaparin        | Tinzaparin        |
| Pre-gestational weight (g)                   | 225.17 ± 22.6     | 226.25 ± 28.1     | 223.33 ± 16.3     | 212.75 ± 12.9     |
| Gestational weight at day 15 (g)             | 291.17 ± 31.7     | 292.25 ± 24.0     | 276.75 ± 15.5     | 272.17 ± 20.8     |
| Litter size                                  | 8.33 ± 1.6        | 7.83 ± 1.9        | 9.00 ± 1.8        | 9.6 ± 1.7         |
| Treatment period (days)                      | 36.33 ± 10.8      | 33.75 ± 5.3       | 33.75 ± 7.0       | 35.25 ± 5.8       |
differ from that in normal third-trimester placenta. In gestational trophoblastic diseases, there was a general trend of down-regulation of both E-cadherin and β-catenin. It has been reported that altered expression of E-cadherin and β-catenin may play a role in the development of normal and pathological placentas. And also in the study by Shih et al. (2002), it has been indicated that expression of E-cadherin in IST-1 cells resulted in a contact-mediated inhibition of motility and invasion and suggest an important role for E-cadherin down-regulation in the intermediate trophoblast during implantation.

Laminins are structurally and functionally major components of the ECM. The ECM is essential for morphogenesis, including cell–cell and cell–matrix interaction and maintenance of all organs and tissues; so, we suggest that laminin may play a role in the development of normal and pathological placentas. And also in the study by Shih et al. (2002), it has been indicated that expression of E-cadherin in IST-1 cells resulted in a contact-mediated inhibition of motility and invasion and suggest an important role for E-cadherin down-regulation in the intermediate trophoblast during implantation.

In conclusion, we suggest that heparin and the LMWH enoxaparin can reduce E-cadherin expression but not laminin expression in rat pregnancy. They might modulate trophoblast invasion. We suggest that this is the possible underlying mechanism involved in the improvement of trophoblast invasion in human placent.

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