ORIGINAL ARTICLE: REPRODUCTIVE BIOLOGY

Effects of low molecular weight heparin and heparin-binding epidermal growth factor on human trophoblast in first trimester

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Objective: To evaluate the effects of low molecular weight heparin (LMWH) and heparin-binding epidermal growth factor (HB-EGF) on the biological function of human trophoblast in first trimester.
Design: Control experiment.
Setting: Two academic-based reproductive centers.
Patient(s): The first trimester human placentas (gestational age, 5–10 weeks) from patients who underwent electively induced abortions.
Intervention(s): Cultured trophoblast treated with LMWH or LMWH and HB-EGF.
Main Outcome Measure(s): The biological function (proliferation, invasion, and differentiation) of trophoblast and the interaction between LMWH and HB-EGF on trophoblast in vitro.
Result(s): 1) At a dose of 0.25 IU/mL–2.5 IU/mL LMWH promoted trophoblast proliferation, enhanced their invasion, and increased hCG secretion. The LMWH had little effect or the opposite effect at other concentrations. These differences were statistically significant. 2) Combined use of LMWH and HB-EGF significantly promoted proliferation and invasion, but there was no difference in hCG secretion compared with solo LMWH or solo HB-EGF.
Conclusion(s): The LMWH exerts a cytoprotective effect by regulating trophoblast proliferation, invasion, and differentiation. The HB-EGF is an important factor in the effects of LMWH on trophoblast function. (Fertil Steril® 2012;■:■–■. ©2012 by American Society for Reproductive Medicine.)
Key Words: Low molecular weight heparin, heparin-binding epidermal growth factor, trophoblast, in vitro

There is mounting evidence that thrombophilia is associated with recurrent spontaneous abortion (RSA) (1, 2). Anticoagulation therapy is recommended to patients with RSA and thrombophilia, among which low molecular weight heparin (LMWH) is the most common treatment (3, 4).

Low molecular weight heparin is preferred to unfractionated heparin because of its better safety profile. It is now the most commonly used anticoagulant for patients with RSA and thrombophilia (4). In 2005, Tzafettas et al. (5) reported that the success rate was high (85.1%) in 27 patients with RSA of unknown etiology after administering LMWH. This revealed that LMWH may protect the embryo by different mechanisms than anticoagulation. Recent evidence shows that there are anti-inflammatory and immunomodulatory effects in LMWH (6–9).

Furthermore, LMWH can regulate the biological function of the human trophoblast. It was reported that LMWH can restore in vitro invasion and differentiation functions of trophoblast inhibited by antiphospholipid antibodies. Evidence suggests that LMWH has a direct influence on trophoblast (10–12).

Heparin-binding epidermal growth factor (HB-EGF) is one of the growth factors that is recognized as having a significant effect on reproduction. It activates two EGF receptor subtypes, HER1 and HER4, and binds heparin sulfate protoglycans to cell surface (13, 14). It has been shown that both decidua and trophocyte express HB-EGF receptors (15, 16). The HB-EGF may promote trophoblast migration and invasion, and therefore improve the survival rate of embryos (15–17). The distinctive structure of HB-EGF means that it can bind to heparin and EGF receptors. Some researchers presume that HB-EGF may play an important role in the effect of LMWH on the trophoblast.

MATERIALS AND METHODS

Grouping

The research has two parts. The first part was to detect the effects of LMWH on the biological function of cultured

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trophoblast, including proliferation, invasion, and differentiation. Each experiment in the first part contained six groups. The study groups included five LMWH groups (treated with enoxaparin at concentrations 0.025, 0.25, 2.5, 25, and 250 IU/mL). The control group was treated with Dulbecco's modified Eagle medium (DMEM) high D-glucose (GIBCO).

The second part was to observe the interaction between LMWH and HB-EGF on trophoblast in vitro and to explore whether LMWH regulated functions of trophoblast through HB-EGF. Four groups were in each experiment: LMWH group (enoxaparin, 0.25 IU/mL), HB-EGF group (10 ng/mL), combination group (enoxaparin 0.25 IU/mL + HB-EGF 10 ng/mL), and control group (DMEM).

Human Placental Tissue Collection in First Trimester Pregnancy

The first trimester human placentas (gestational age, 5–10 weeks) were obtained from clinically normal pregnancies that were terminated for nonmedical reasons at the Second Affiliated Hospital of Sun Yat-sen University. Each patient completed a signed, written consent form. All the tissues were put immediately into ice-cold DMEM high D-glucose (GIBCO), transported to the laboratory within 30 minutes after surgery and washed in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) for trophoblast isolation.

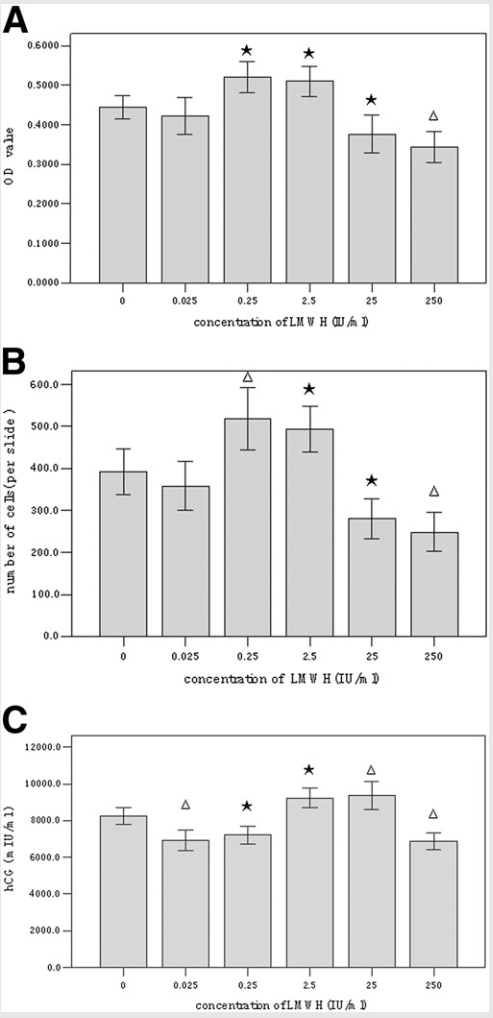
Isolation and Primary Culture of the First Trimester Human Trophoblast Cells

The first trimester human placenta was separated carefully from the decidua under a stereomicroscope, minced into small fragments, and digested in DMEM (GIBCO) containing 0.25% trypsin (Bio Basic Inco) and 0.02% DNase type I (Sigma) for 10 minutes at 37°C. The trypsin digestion was stopped with 10% fetal calf serum (FCS) (Hyclone). The remaining placental tissues were subject to another three cycles of 10-minute trypsinization. Then the suspension was filtered through a stainless steel mesh of 100 μm pores. The resultant cell suspensions were pooled, centrifuged at 1,000 rpm for 10 minutes, and resuspended in 3 mL of DMEM (GIBCO). This suspension was layered over a preformed Percoll gradient (Pharmacia) made up in DMEM. The gradient was made from 65%–25% Percoll (vol/vol) in 5% steps of 2 mL each. The gradient was centrifuged at 2,000 rpm for 20 minutes. The middle layer (density, 1.048–1.062 g/mL) was removed and washed with DMEM (GIBCO). Cells were cultured in DMEM high glucose supplemented with 20% FBS (GIBCO), 2 mM glutamine, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 100 UI/mL penicillin, and 100 μg/mL streptomycin at 37°C in 5% CO_2.

Immunocytochemistry

After 24 hours of culture, the trophoblast cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature, washed in phosphate-buffered saline (PBS), and permeabilized for 30 minutes in 0.3% Triton X-100 PBS. The cells were incubated with 3% H_2O_2, away from light. Cells were incubated with 7% goat serum in PBS for 30 minutes to reduce nonspecific binding. Primary antibodies diluted in PBS containing 1% bovine serum albumin (BSA) were added. Anti-human cytokeratin-7 and anti-human vimentin monoclonal antibody (mAb) (Sino-America Co. Ltd.) were used as markers for identification of trophoblast lineage and nontrophoblast lineage. Isotype-matched irrelevant IgG (Sino-America Co. Ltd.) was used as a control. After incubation with primary mAb overnight at 4°C, the cells were washed in PBS, and then incubated with horseradish peroxidase-labeled secondary mAb (Sino-America Co. Ltd.) for 2 hours at room temperature. The slides were stained with 3, 3-diaminobenzidine and counterstained with hematoxylin. The experiments were repeated three times.

FIGURE 1



(A) Effects of low molecular weight heparin (LMWH) on trophoblast proliferation. Error bars depict SEM. (B) Effects of LMWH on trophoblast invasion. Error bars depict SEM. (C) Effects of LMWH on trophoblast hCG levels. Error bars depict SEM. ★ Compared with group 0 $P<.05$; Δ compared with group 0 $P<.01$.

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MTT Test

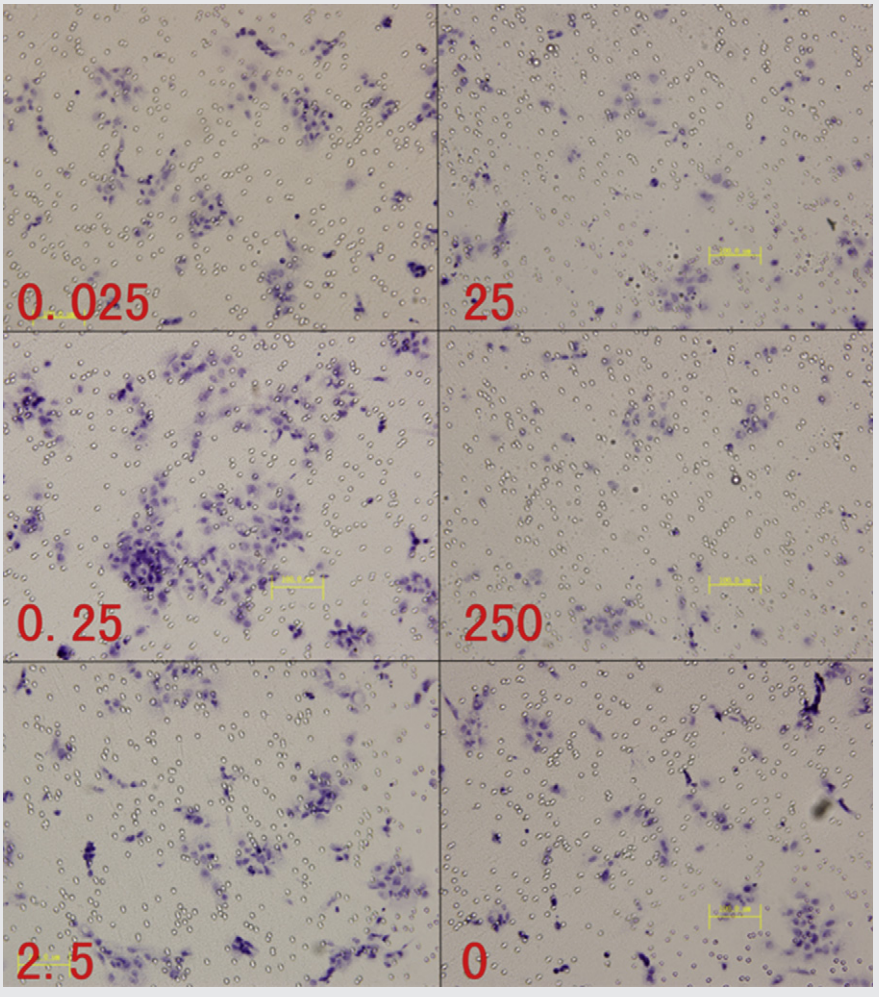
Trophoblast cells (2×10^4 per well) were added to 96-well microtiter plates in 100 μL/well of medium and cultured in

DMEM supplemented with 20% FBS at 37°C in an atmosphere containing 5% CO_2. After overnight incubation, the medium was replaced with DMEM supplemented with 1% FBS for another 12 hours. Then various concentrations of enoxaparin ("clexane," Sanofi-aventis) and HB-EGF (R&D Systems) in the presence of DMEM were supplemented for 44 hours. At the end of this incubation, 20 μL of 93-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (Sigma) (MTT) (5 mg/mL) was added into each well, and trophocytes were cultured for an additional 4 hours. Finally, the medium was removed, and 150 μL of dimethyl sulfoxide (DMSO) was added and shaken for 10 minutes. Absorbency was measured at a wavelength of 490 nm on an automatic microplate reader. The absorbance change was then converted to cell number, based on a calibration with the known cell number of 490 nm. All experiments were performed three times or more with fresh cultures.

Invasion Assay

Trophoblast cells were cultured on Matrigel in 6.4-mm transwell inserts with polycarbonate membrane filters containing 8-μm pores (Corning). To prepare the gel, 30 μL of Matrigel, diluted with DMEM (1:2), was incubated on top of the membranes at 37°C and the inserts were placed into 24-well culture plates. After gel formation, trophoblast cells (1×10^5 per well) were added into the upper compartment in 100 μL/well of DMEM supplemented with 1% FBS. The LMWH and HB-EGF were added, reaching the presumed final concentration. Then 800 μL of DMEM with 10% FBS was added into the lower compartment. The trophoblast cells were incubated at 37°C in an atmosphere containing 5% CO_2. After being cultured for 48 hours, the cells adhering to the supine surface of the upper compartment were lightly wiped using cotton buds, whereas the cells migrating to the inferior surface of the microporous membrane were fixed by 4% paraformaldehyde, dyed with

FIGURE 2



Effects of low molecular weight heparin on trophoblast invasion. The invasive index of cells under different conditions was normalized to the control. Microscopic morphologies of the invasion of trophoblasts through the matrigel-coated membranes were taken at magnification ×200. Trophoblast was incubated in the presence of 0.025 IU/mL enoxaparin (0.025), 0.25 IU/mL enoxaparin (0.25), 2.5 IU/mL enoxaparin (2.5), 25 IU/mL enoxaparin (25), 250 IU/mL enoxaparin (250), and control (0).

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hematoxylin and mounted with nertrogum. The number of cells per pore was counted in five randomly selected fields with a microscope. The cell numbers in each pore were assessed according to Image-Pro Plus 6.0 (Media Cybernetics). All experiments were performed three times or more with fresh cultures.

hCG Secretion Test

The hCG secretion test was designed to explore the effects of LMWH and HB-EGF on trophoblast differentiation. The human trophoblast cells (3×10^5 per well) were plated out in 24-well plates and cultured overnight. The LMWH at different concentrations and HB-EGF in the presence of LMWH were added to fresh mediums. After incubated for 48 hours the supernatant was collected and stored at $-20°C$. The concentration of β-hCG in the supernatant was determined using the chemiluminescence method. All experiments were performed three times or more with fresh cultures.

Statistical Analysis

All values were expressed as the mean ± SD. Data were analyzed using one-way analysis of variance (ANOVA) with the application of the Dunnett's test, the least-significant difference test, and a three-factor ANOVA classification. Differences were considered as statistically significant at $P<.05$.

RESULTS

Isolation, Identification, and In Vitro Culture of Human Trophoblast

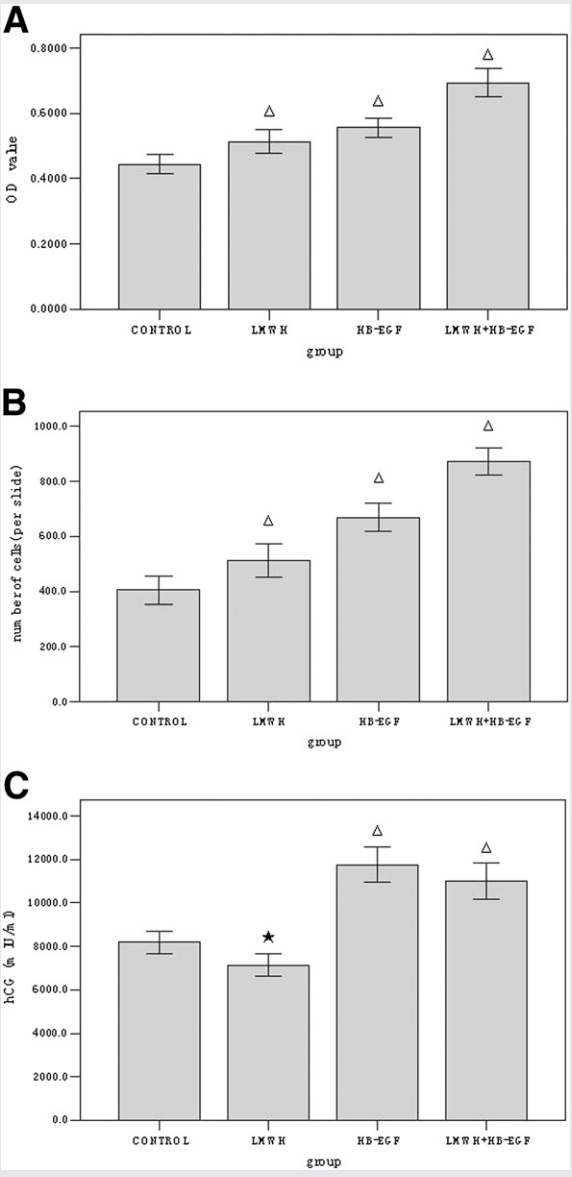
The immunocytochemistry results showed that trophoblasts exhibited positive staining for cytokeratin 7, but negative staining for vimentin. Although a few spindle cells were found to positively express vimentin, they were considered to be fibroblasts. The immunocytochemistry also indicated that the purity of isolated trophoblast cells was more than 90%.

Effects of LMWH on Biological Functions of Trophoblast Cells

As concentrations increased, the influence of LMWH on trophoblast cells changed. The 0.025 IU/mL LMWH had little effect on trophoblast proliferation and invasion ($P>.05$); within the doses of 0.25–2.5 IU/mL, LMWH greatly promoted trophoblast proliferation and enhanced their invasion ($P<.05$); however, proliferation and invasion of the cells was suppressed if the concentration increased from 25–250 IU/mL (Figs. 1A and B and 2).

Low molecular weight heparin also had an influence on hCG secretion of trophoblast. At the therapeutic dose (0.025–0.25 IU/mL), LMWH greatly inhibited hCG secretion ($P<.05$). With the increased concentration of LMWH, the inhibition of LMWH on hCG secretion shrank correspondently, but the difference was not significant ($P>.05$). At the concentration of 2.5–25 IU/mL, LMWH significantly increased hCG secretion ($P<.05$). Meanwhile, 250 IU/mL LMWH inhibited hCG secretion ($P<.05$) (Fig. 1C).

FIGURE 3



(A) Effects of low molecular weight heparin (LMWH) and heparin-binding epidermal growth factor (HB-EGF) on trophoblast proliferation. Error bars depict SEM (Δ compared with group control $P<.01$). (B) Effects of LMWH and HB-EGF on trophoblast invasion. Error bars depict SEM (Δ compared with group control $P<.01$). (C) Effects of LMWH and HB-EGF on hCG levels. Error bars depict SEM (★ compared with group control $P<.05$; Δ compared with group control $P<.01$).

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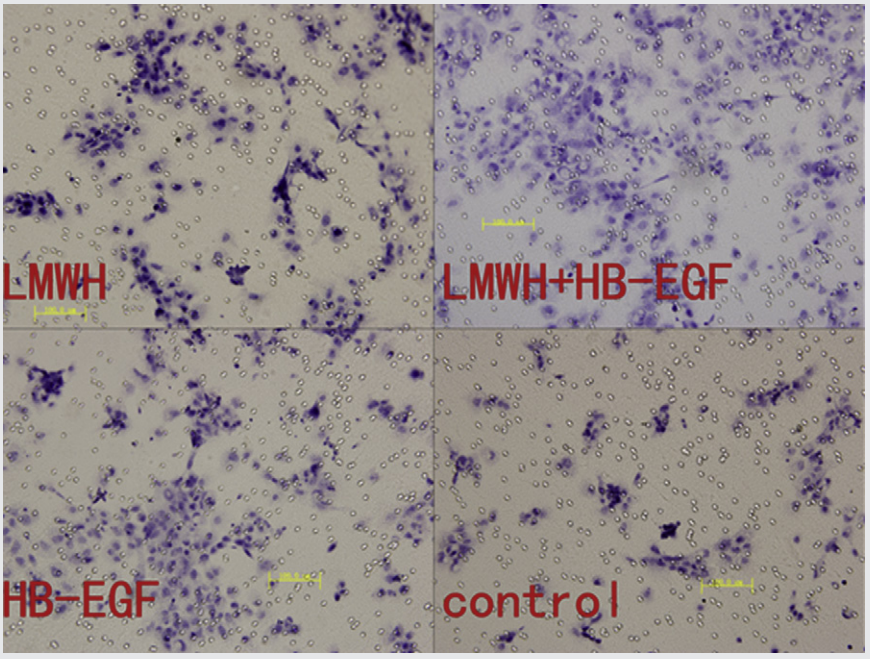
This test showed that there was an interaction between LMWH and HB-EGF on trophoblast proliferation and invasion when they were simultaneously applied ($P<.05$). Compared with either solo LMWH or HB-EGF, simultaneous application of these two agents was found to significantly promote trophoblast proliferation and invasion ($P<.05$). Compared with the control group, solo LMWH, and solo HB-EGF, simultaneous application could promote proliferation and invasion ($P<.05$) (Figs. 3A and B and 4).

FIGURE 4



Effects of low molecular weight heparin (LMWH) and heparin-binding epidermal growth factor (HB-EGF) on trophoblast invasion. The invasive index of cells under different conditions was normalized to the control. Microscopic morphologies of the invasion of trophoblasts through the matrigel-coated membranes were taken at magnification ×200. Trophoblast was incubated in the presence of LMWH (0.25 IU/mL enoxaparin), HB-EGF (10 ng/mL HB-EGF), LMWH + HB-EGF (0.25 IU/mL enoxaparin + 10 ng/mL HB-EGF), and control (Dulbecco's modified Eagle medium [DMEM]).

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No interaction between LMWH and HB-EGF was found with regard to hCG secretion when they were simultaneously applied in vitro ($P>.05$). Compared with the control group, the inhibition of solo LMWH on hCG secretion was significant ($P<.05$). The promotion of solo HB-EGF and simultaneous application on hCG secretion was also significant ($P<.05$). However, there was no difference between the effect of solo HB-EGF and simultaneous application on hCG secretion ($P>.05$) (Fig. 3C).

DISCUSSION

LMWH and Trophoblast

Proliferation of human trophoblast is a physiological process, and it is important for remodeling of tissue at the maternal–fetal interface. Our results showed that LMWH exerts a dual effect on trophoblast proliferation, characterized by promotion of cell proliferation at low concentrations (0.25–2.5 IU/mL) and inhibition at high concentrations (25–250 IU/mL). A range of 0.05–0.25 IU/mL was established in the patients receiving prophylactic heparin dosing (18). Quenby et al. (10) demonstrated that the therapeutic doses of unfractionated heparin and LMWH were 0.025–0.25 IU/mL.

There was a similar tendency of LMWH on trophoblast invasion. At therapeutic doses (0.25–2.5 IU/mL), LMWH promoted trophoblast invasion, but it suppressed if the concentration reached 25–250 IU/mL. However, not all researchers got the same results. Ramesh et al. (12) found that fractionated heparin reduced invasion in the SGHPL4 cell line and in placental tissue in a dose-dependent fashion. Di Simone et al. (11) reported that LMWH induced in vitro trophoblast invasiveness. Quenby et al. (10) reported that at a therapeutic dose of 0.25 IU/mL, LMWH had no effect on trophoblast motility, but at supratherapeutic doses it inhibited cell motility. The contradiction may come from the differences of experimental methods, materials, cases included, and so on.

As we know, during early human pregnancy, failure of trophoblast invasion always leads to pregnancy-complicated diseases, such as pre-eclampsia and fetal growth restriction (19, 20). In contrast to tumoral invasiveness, trophoblast invasion is precisely regulated and controlled. Many studies have indicated that LMWH was one of the factors regulating trophoblast invasion, although the results were not always consistent. In 2006, Erden et al. (21) found the underlying mechanism involved in the improvement of trophoblast invasion by use of LMWH in patients with a history of miscarriage. They reported that enoxaparin can reduce E-cadherin expression but not laminin expression in rat pregnancy, which might modulate trophoblast invasion. Also, Di Simone et al. (11) pointed out the effective role of matrix metalloproteinases and tissue inhibitors in the modulation of LMWH on trophoblast invasion.

The cytotrophoblasts on the border layer of the floating villi differentiate by cell–cell fusion into multinucleate syncytiotrophoblasts, which cover floating villi, and mediate substance exchange between fetus and mother. In addition, they exert endocrine function. A small amount of hCG can be secreted by syncytiotrophoblasts starting on the sixth

day after fertilization and then it increases rapidly during the first trimester. The secretion of hCG is considered to be an important sign of trophoblast differentiation. It appears that hCG autoregulates trophoblast function (22). Low-to-moderate hCG concentration produced by placenta can stimulate differentiation to increase its own synthesis. When sufficiently high hCG levels are reached, differentiation is inhibited to prevent an uncontrolled increase in hCG levels (23, 24). In addition to its function in the regulation of differentiation, hCG stimulates the migration and invasion of trophoblast in vitro (25, 26). But the mechanism of hCG on trophoblast invasion is still not fully understood.

Our results found that LMWH also had an influence on trophoblast hCG secretion. At therapeutic doses, LMWH greatly inhibited trophoblast differentiation. With the increased concentration of LMWH, the inhibition of LMWH on hCG secretion shrank correspondently. At supratherapeutic concentration (2.5–25 IU/mL), LMWH significantly increased hCG secretion. Furthermore, 250 IU/mL LMWH decreased hCG secretion. Because the autoregulation mechanism of hCG is poorly understood, and there are many factors in vivo affecting hCG levels, we cannot explain the exact role of LMWH on hCG secretion at therapeutic doses, or the consequences for pregnancy outcomes.

HB-EGF and Trophoblast

The HB-EGF is one of the growth factors that are now recognized as having significant functions in reproduction. It can be expressed as biologically active soluble and transmembrane forms (13).

During pregnancy, villous cytotrophoblasts and extravillous cytotrophoblasts express HB-EGF, which is thought to be an important factor in fetal development. Leach et al. (15) found that the addition of HB-EGF during the explant culture of first trimester chorionic villi enhanced extravillous trophoblast invasive activity. Lala and Chakraborty (27) reported that HB-EGF promoted adhesion and outgrowth of mature blastocysts on matrigel, whereas impaired HB-EGF signaling during trophoblast development contributed to impaired invasiveness and increased apoptosis in the pre-eclamptic trophoblast. Also, Fisher and Lakshmanan (28) proved that HB-EGF can accelerate trophoblast differentiation because of its promotion on the hCG and human placental lactogen secretion of trophoblast.

Our results showed that HB-EGF (10 ng/mL) facilitated proliferation, invasion, and differentiation of human trophoblast, which was consistent with the results of other researchers. Also, there were interactions between LMWH and HB-EGF on trophoblast proliferation and invasion when they were simultaneously applied in vitro. However, no interaction was found on trophoblast differentiation. We presume that HB-EGF was an important factor in the effects of LMWH on trophoblast but not the exclusive factor.

Together, the data strongly suggest that LMWH exerts its cytoprotective effect by regulating trophoblast proliferation, invasion, and differentiation. But the effects can be influenced by other factors, such as the drug concentration and starting time. The interactions between LMWH and HB-EGF may show that HB-EGF is an important factor in the effects of LMWH on trophoblast functions. This research may influence more extensive application of LMWH on RSA, and its use as a potential medicine for the pregnancy-complicated diseases.

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