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From Cell Biology to Tissue Engineering

Human trophoblast cell during first trimester after IVF-ET differs from natural conceived pregnancy in development and function

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Summary. Objective. To explore the differences of the trophoblast cell function in first trimester between natural pregnancy and pregnancy after IVF-ET therapy.

Methods. 102 cases with twin to singleton fetal reduction after IVF-ET treatment from July 2010 to August 2013 in Peking University Third Hospital were involved in analysis, and eight specimens were obtained from this group. 10 natural-pregnancy cases undergoing artificial abortion with unwanted pregnancy were recruited as control. Semi-quantitative immunohistochemical method was used to detect the expression of EGFR, Bcl-2, tubulin-α, metallothionein and AFP in villi in both groups.

Results. Of the 102 cases, 14 cases (13.73%) were aborted. Preterm birth occurred in seven cases (7.86%). Low birth weight occurred in three patients (3.37%), and extremely low birth weight occurred in four cases (4.49%). The expression of EGFR, tubulin-α, Bcl-2, and metallothionein in the IVF-ET group was significantly lower than that in the control group ($P<0.05$). However, AFP expression was significantly higher in IVF-ET group than in control group ($P<0.05$). In IVF-ET group, the miscarriage case had weaker EGFR, tubulin-α, and metallothionein expression than full-term pregnancy; the early preterm labor case had weaker Bcl-2, tubulin-α, and metallothionein expression; and velamentous cord insertion case had weaker tubulin-α expression.

Conclusions. The trophoblast cell function of IVF-ET group in first trimester is different from control group in proliferation, invasion, apoptosis and vascular development, and optimal pregnancy outcome depends on the self-healing balance of trophoblast cells.

Key words: Cytotrophoblast cell, Syncytiotrophoblast cell, *In vitro* fertilization and embryo transfer, Natural pregnancy, Proteomic analysis

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Introduction

Therapeutic methods for infertility caused by various etiological factors have improved greatly in recent years, particularly assisted reproductive technologies (ART). *In vitro* fertilization and embryo transfer (IVF-ET) have resulted in a revolution in infertility treatment. However, much clinical literature (Rimm et al., 2009; Klatsky et al., 2010; Calhoun et al., 2011; Tsoumpou et al., 2011; Sazonova et al., 2012) has reported that IVF-ET technology may be closely related to many adverse outcomes during the perinatal period, including miscarriage, premature birth, low birth weight, and gestational hypertension.

The key to normal embryo implantation and pregnancy lies in the preferential invasion of early pregnancy trophoblastic cells into the uterine wall. Dysregulation of this invasion process can result in a series of adverse outcomes, such as premature delivery, placenta previa, low birth weight, and gestational hypertension (Norwitz et al., 2007). Zhang et al. (2008) reported that IVF manipulation may affect fetal development by changing maternal-fetal immuno-

reactions and nourishing cell proliferation and differentiation, or by impairing transmembrane transport. However, few studies have explored whether these abnormalities occur during early or late pregnancy.

The aim of this study was to explore the markers of trophoblast function to predict the pregnancy outcome after IVF-ET, and to compare the relationships between the functional changes of trophoblast cells and the pregnancy outcomes during the early stages of IVF-ET pregnancy and natural pregnancy.

Materials and methods

Material source

Twin to singleton fetal reduction was performed in a total of 102 cases after IVF-ET treatment from July 2010 to August 2013, and eight specimens were obtained from the observation group (age range: 23-35 years; mean age: 30.8 years) at the Reproductive Medicine Center for Male or Oviducts Factors at Peking University Third Hospital, Beijing, China. The reasons for fetal reduction on the 102 cases from twin to singleton in this study include: chronic diseases (such as hypertension), uterine malformation (such as single horn uterine), scarred uterine (myomectomy history or Caesarean history), uterine leiomyoma, cervical incompetence, cervical conization history, or patients' demand.

All the fetal reductions were performed by the same senior physician through fetal bud aspiration under B ultrasound guidance. 42 cases did fetal reduction through fetal bud aspiration in a total 102 cases, and we selected 8 cases for villi suction at the same time. Aspiration could not be successfully performed on the other 60 patients for whom we used KCl local injection instead. The tissue was collected 30-45 days after embryo transfer, which is equivalent to 45-50 days of pregnancy.

The control group included 10 cases of unwanted singleton pregnancy with a mean of 49±6 days of pregnancy from June 2013 to August 2013 (age range: 24-30 years; mean age: 28.9 years). All the patients had regular menstruation and had not taken any steroid hormone drugs in the previous 3 months. Further, all the patients were diagnosed with early intrauterine pregnancy after bimanual examination, urine pregnancy test, and B ultrasound. The villi were obtained during the conventional artificial abortion operation.

The study was approved by the Ethics Committee of Peking University Third Hospital and all patients signed written informed consents. Full-thickness villi 1.0×1.0 cm in size were removed from the specimens within 1 h after collection. After rinsing with saline, the villi were immersed in 4% formalin for 24 h and then taken out and rinsed with running water for 30 minutes. The villi then underwent routine dehydration, wax dipping, embedding, and slicing (4 μm thick). One slice was obtained from each sample and was stained via two-step immunohistochemical PV-9000.

Immunohistochemistry

Antibodies

Mouse anti-human epidermal growth factor receptor (EGFR) monoclonal antibody solution (ZA-0093; Zhongshan Golden Bridge Biotechnology Co., Ltd., Zhongshan, China); mouse anti-human B-cell lymphoma-2 (Bcl-2) protein monoclonal antibody solution (ZA-0010; Zhongshan Golden Bridge Biotechnology Co., Ltd.); mouse anti-human α-1-fetoprotein (AFP) monoclonal antibody solution (ZM-0009; Zhongshan Golden Bridge Biotechnology Co., Ltd.); rabbit anti-human tubulin-α polyclonal antibody solution (ZA-0435; Zhongshan Golden Bridge Biotechnology Co., Ltd.); mouse anti-human metallothionein monoclonal antibody solution (ZA-0188; Zhongshan Golden Bridge Biotechnology Co., Ltd.).

Reagents

PV-9000 two-step immunohistochemical kit (Zhongshan Golden Bridge Biotechnology Co., Ltd.); DAB chromogenic kit (20×) (Z61-9018; Zhongshan Golden Bridge Biotechnology Co., Ltd.); antibody diluent (ELI-9029; Zhongshan Golden Bridge Biotechnology Co., Ltd.); phosphate buffered saline (PBS) (10×), diluted with distilled water to 1 × sodium citrate antigen retrieval solution (2×), diluted with distilled water to 1×, pH 6.0; EDTA antigen retrieval solution (50×), diluted with distilled water to 1×, pH 8.0. Experimental procedure: two-step immunohistochemical PV-9000 method; slice dewaxing: immersion in xylene for 5 min ×2; gradient alcohol to water; antigen retrieval.

Hot water retrieval

The slice was immersed in EDTA antigen retrieval solution (EGFR) at pH 8.0, sodium citrate antigen retrieval solution (Bcl-2, tubulin-α) pH 6.0, and 98°C water for 20 min; metallothionein and AFP without retrieval; added 3% H_2O_2 solution into the sliced tissue, incubated at room temperature for 10 min, washed them once with distilled water and three times with PBS for 3 min each time, and then followed by the addition of the first antibody as mentioned above. PBS was used to replace the first antibody as the negative control, and all the samples were incubated overnight at 4°C. The sections were incubated with the second antibody for 30 minutes. Diaminobenzidine was used as a chromogen, and sections were counterstained with hematoxylin.

Assessment of the immunohistochemical staining results

The immunohistochemical staining of the villi in the slices was observed using optical microscopy (Eclipse 80i; Nikon Corp., Tokyo, Japan). The positive reaction products were brown-yellow granules. Metallothionein,

AFP and tubulin-α were located in the cytoplasm; Bcl-2 and EGFR were located in the cell membrane and cytoplasm.

Image-Pro Plus analysis software was used on the immunohistochemical data for semi-quantitative analysis of the staining intensity. The images were enlarged 400 times using the double-blind method and ordinary illumination, and five areas from each slice were randomly selected on the high-resolution monitor. The mean optical density (MOD) of positive expression was measured in each area, and the mean was calculated to obtain an MOD that was representative of the slice. Five slices from each sample were assessed, and the mean for each group was calculated.

Statistical analysis

SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA) was used for the collection, processing, and statistical analysis of the data. The data are expressed as the means ± standard errors, and statistical analysis was performed using independent samples t-tests for comparison of the means. P-values less than 0.05 were considered statistically significant.

Results

Characteristics of the patients in the two groups and tissue hematoxylin and eosin (H.E.) staining

There were no statistical differences between the two groups of women in terms of age, gestational age, and body mass index (Table 1). Villi were observed (arrow) in both groups (see Fig. 1).

EGFR expression in villi in both groups

The expression of EGFR in the placental villi in the IVF-ET group was lower than that in the control group (see Fig. 2; $P<0.05$). EGFR is widely distributed in the cytoplasm and cytomembrane of villous syncytiotrophoblast and trophoblast cells during early pregnancy, the former of which has higher expression. In the IVF-ET group, EGFR expression in the villous syncytiotrophoblasts was significantly lower, whereas it was higher in the trophoblast cells.

Table 1. Characteristics of the patients in the two groups.

	N	Mean age	Gestational age (day)	Gravidity	BMI (kg/m^2)
Observation group	8	30.75±3.96	48.38±3.54	1.00±1.31	24.38±4.08
Control group	10	28.90±3.00	49.10±2.23	2.10±0.99	22.89±2.05
P-value		0.275	0.603	0.059	0.369

BMI, body mass index.

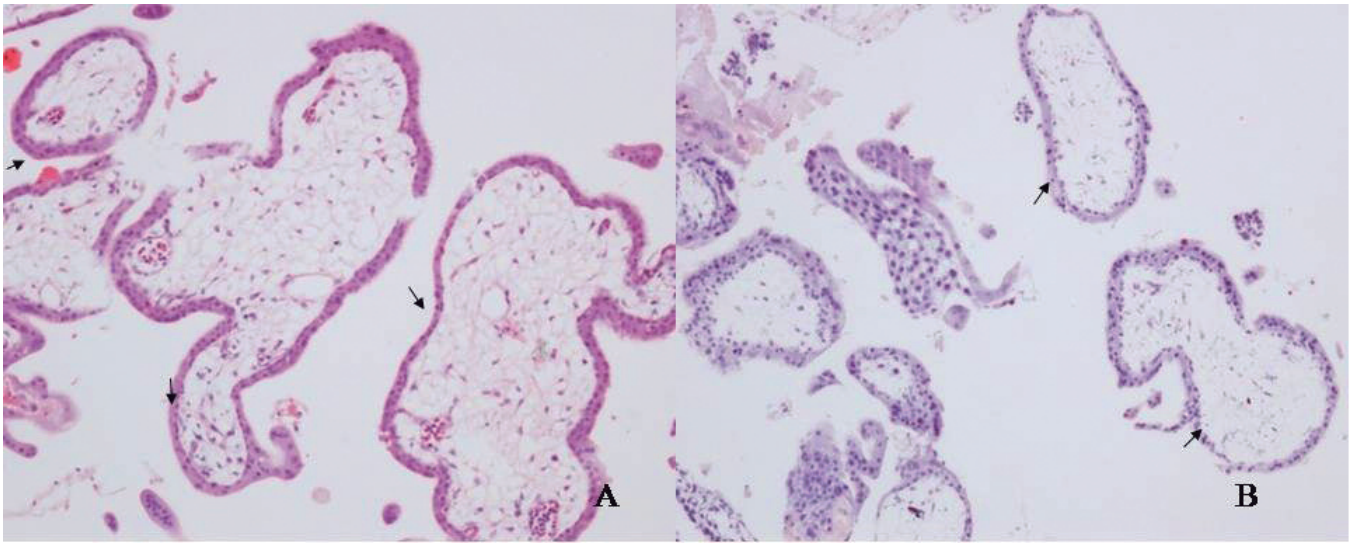

Fig. 1. Villi were observed (arrows) in both groups (H.E. staining). **A.** Control group. **B.** IVF-ET group. H.E. × 100

Tubulin-α expression in villi in both groups

The expression of tubulin-α in the villi tissue of women in IVF-ET group was significantly lower than that in control group (see Fig. 3; $P<0.05$). Tubulin-α is located in the plasma of syncytiotrophoblast and cytotrophoblast cells of early villi, and it was more strongly expressed in the cytotrophoblast cells but not in syncytiotrophoblast cells in IVF-ET group. In the control group, tubulin-α expression was strong in both syncytiotrophoblast and cytotrophoblast cells.

Bcl-2 expression in villi in both groups

The expression of Bcl-2 in the placental villi in the IVF-ET group was lower than that in control group (see Fig. 4; $P<0.05$). Bcl-2 is widely distributed in the cytoplasm of villous syncytiotrophoblasts and in the

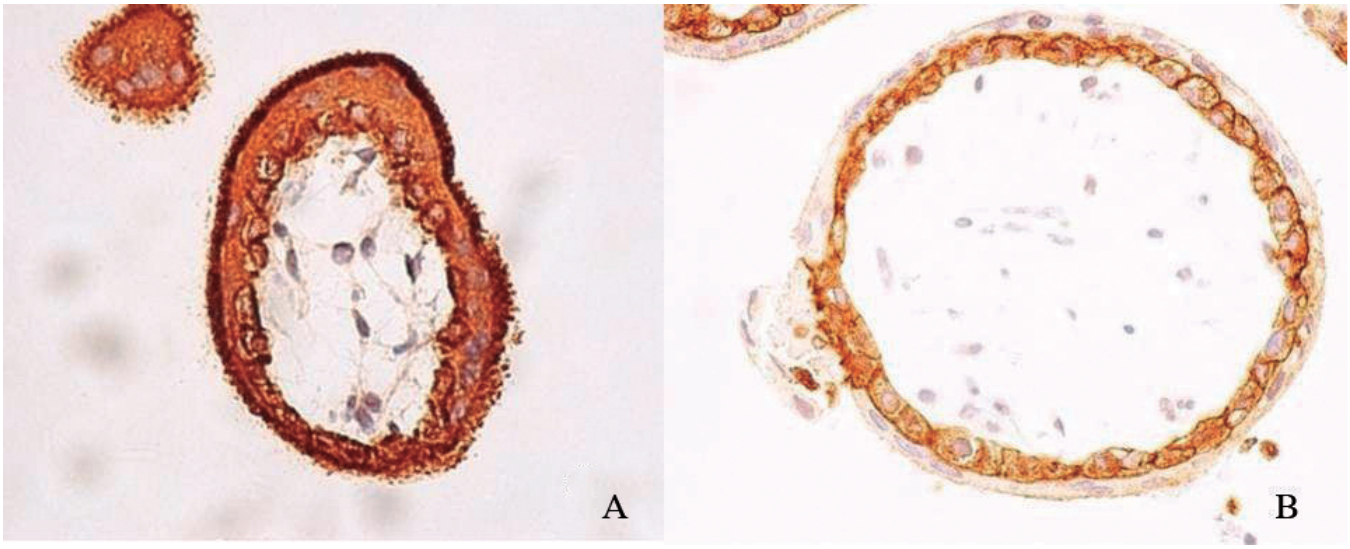

Fig. 2. EGFR had high positive expression in the control group **(A)**, and low positive expression in the IVF-ET group **(B)**. EGFR, epidermal growth factor receptor; IVF-ET, *in vitro* fertilization and embryo transfer. x 400

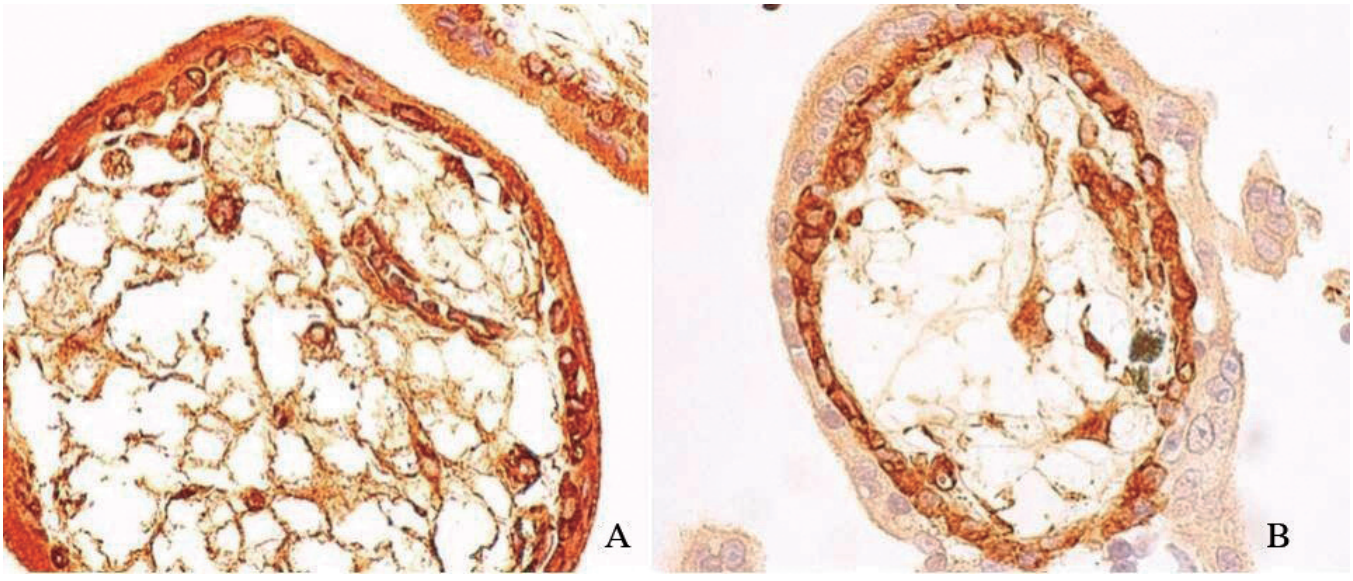

Fig. 3. A. Tubulin-α was strongly expressed in early villi in the control group. **B.** Low positive expression of tubulin-α was observed in trophoblastic cells but not syncytiotrophoblast cells in IVF-ET group. × 400

cytomembrane of trophoblast cells during early pregnancy. In the IVF-ET group, low expression of Bcl-2 was observed in both kinds of cells.

Metallothionein expression in villi in both groups

The expression of metallothionein in the villi tissues of women in IVF-ET group was significantly lower than that in control group (see Fig. 5; $P<0.05$). Metallothionein is found in the plasma of syncytiotrophoblast and cytotrophoblast cells and is more strongly expressed in cytotrophoblast cells, and its expression was significantly lower in both types of cells in the IVF-ET group.

AFP expression in villi in both groups

The expression of AFP in the placental villi in the IVF-ET group was significantly higher than that in control group (Fig. 6; $P<0.05$). AFP is found in the

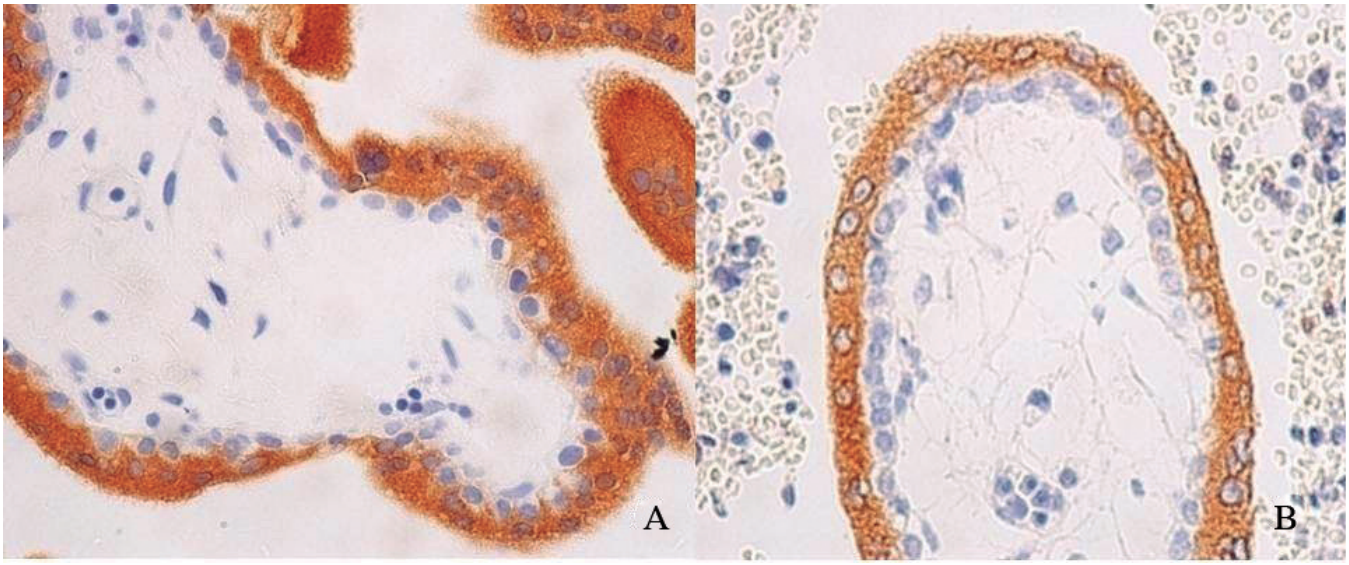

Fig. 4. Bcl-2 had high positive expression in the control group **(A)**, and low positive expression in IVF-ET group **(B).** × 400

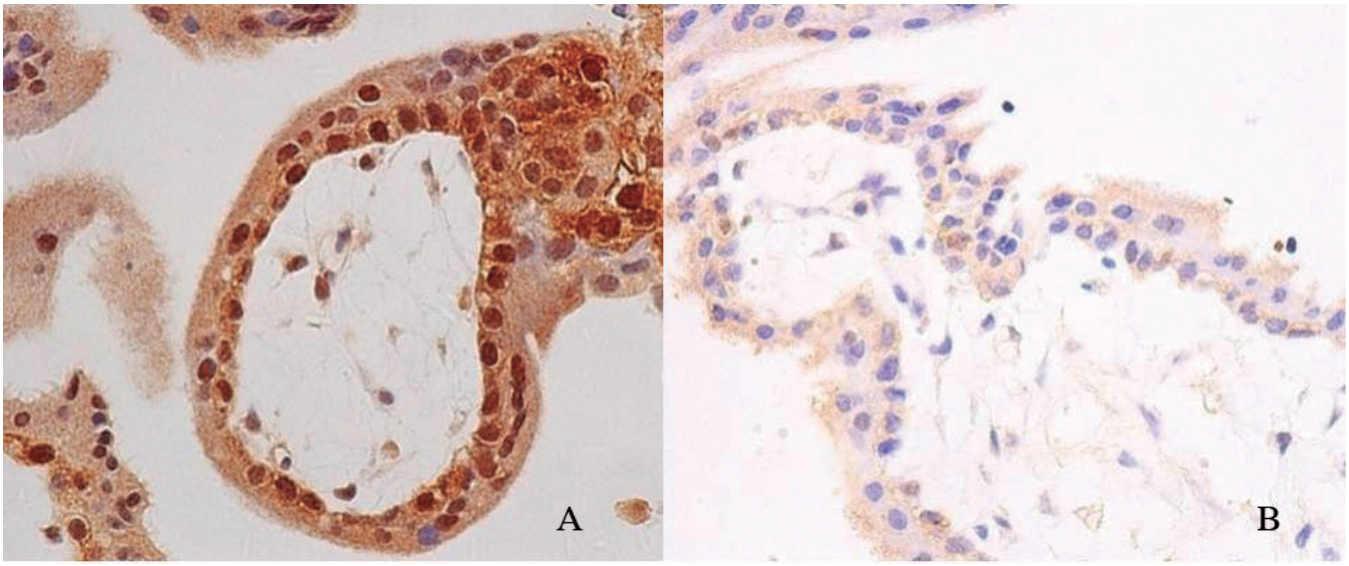

Fig. 5. A. Metallothionein was strongly expressed in the early villi in the control group. **B.** low positive expression of metallothionein was observed in the early villi in IVF-ET group. × 400

cytoplasm of villous syncytiotrophoblasts but not in cytotrophoblast cells during early pregnancy. AFP was expressed in both villous syncytiotrophoblast and trophoblast cells in the IVF-ET group, whereas there was only low expression in the villous syncytiotrophoblasts in the control group.

MOD of the two groups in villi in both groups

The expression of EGFR, tubulin-α, Bcl-2, and metallothionein in the placental villi in the IVF-ET group was significantly lower than that in the control group (P<0.05); however, AFP expression was

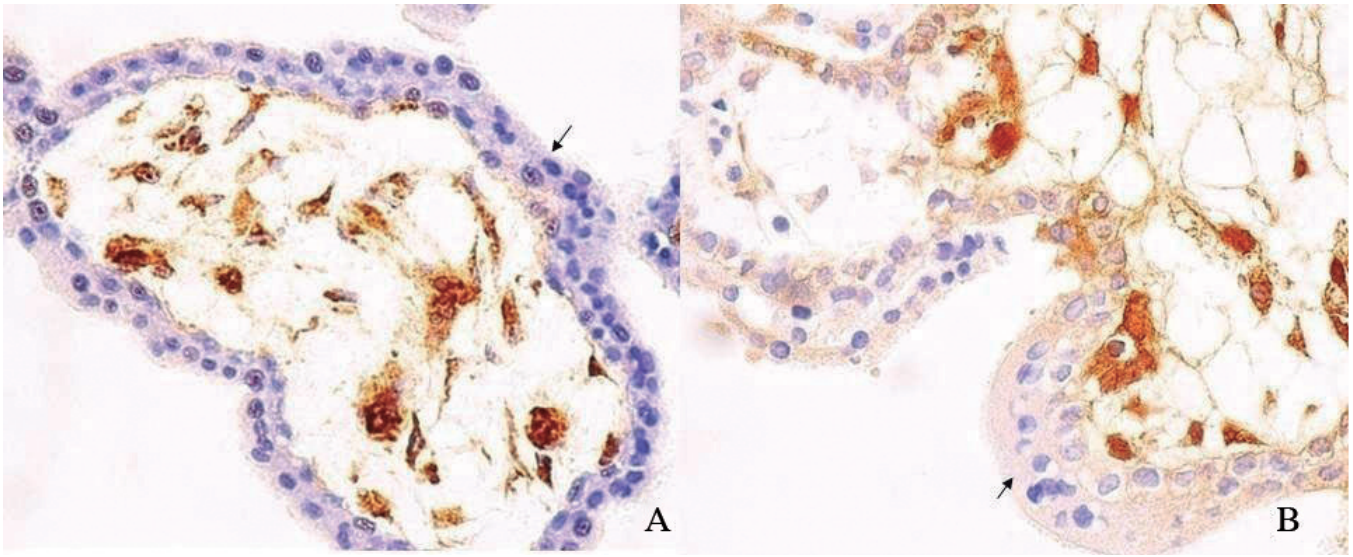

Fig. 6. A. AFP was negatively expressed in the control group. **B.** low positive expression of AFP was observed in IVF-ET group. × 400

Table 2. MOD of positive expression in the two groups (OD, $\bar{\chi}\pm s$).

	IVF-ET group		Control group		P-value
	N	$\bar{\chi}\pm s$	N	$\bar{\chi}\pm s$	
EGFR	8	0.072±0.011	10	0.086±0.009	0.008
Tubulin-α	8	0.024±0.009	10	0.040±0.011	0.003
Bcl-2	8	0.036±0.004	10	0.040±0.004	0.013
Metallothionein	8	0.084±0.012	10	0.098±0.014	0.037
AFP	8	0.067±0.005	10	0.061±0.004	0.008

MOD, mean optical density; OD, optical density; EGFR, epidermal growth factor receptor; AFP, α-1-fetoprotein.

Table 3. The MOD of the immunohistochemistry results from eight patients who underwent fetal reduction and the outcome of the remaining embryonic pregnancies.

Patient	EGFR	Tubulin-α	Bcl-2	Metallothionein	AFP	Pregnancy outcome
1	0.056	0.012	0.038	0.072	0.068	Miscarriage
2	0.062	0.016	0.030	0.064	0.070	Preterm labor at 28 weeks
3	0.072	0.015	0.045	0.086	0.066	Term birth Velamentous cord insertion
4	0.082	0.024	0.034	0.097	0.063	Term birth
5	0.068	0.030	0.036	0.079	0.061	Term birth
6	0.081	0.028	0.034	0.091	0.073	Term birth
7	0.069	0.038	0.034	0.098	0.062	Term birth
8	0.088	0.029	0.038	0.087	0.071	Term birth

MOD, mean optical density; EGFR, epidermal growth factor receptor; AFP, α-1-fetoprotein.

significantly higher in IVF-ET group than in control group (P<0.05) (Table 2).

Pregnancy outcome in the IVF-ET group in both groups

Of the 102 cases, 14 cases (13.73%) were aborted, six of which were early onset cases and eight of which were late onset cases (two late onset cases with fetal malformation). Preterm birth occurred in seven cases (7.86%) (28-36 gestational weeks). No birth defects were observed, and the mean birth weight was 3165.4 g. Low birth weight (2000-2500 g) occurred in three patients (3.37%), and extremely low birth weight (1000-2000 g) occurred in four cases (4.49%).

Of the eight patients from which the specimens were obtained, one patient had a miscarriage at week 9, and one went into preterm labor at week 28, with the newborn weighing 1050 g. The other six infants were delivered by cesarean section at term. The mean weight of the six newborns was 3550 g. Velamentous cord insertion was observed for one case but no other pregnancy complications were observed such as gestational hypertension or gestational diabetes.

The immunohistochemical staining of the villus from Patient 1 (miscarriage) showed that the expression of EGFR, tubulin-α, and metallothionein was weaker than in the other six cases, which reached full-term pregnancy. The staining intensity of Bcl-2, tubulin-α, and metallothionein was very weak in Patient 2 (preterm labor). Patient 3 (velamentous cord insertion) had weak tubulin-α staining intensity (see Table 3).

Discussion

Successful embryonic implantation is dependent on a complex interplay between the invading trophoblast and the maternal tissue. Trophoblast cells are derived from cells in the outer layer of early embryos or blastocysts. On the seventh day after fertilization, trophoblasts proliferate and then differentiate into interstitial trophoblast cells and vascular endothelial trophoblast cells. Vascular endothelial trophoblast cells infiltrate the spiral arteries, replacing part of the vascular endothelium (Whitley and Cartwright, 2009), and then substitute the medial smooth muscle or elastic tissue in vessels with a fiber-like substance. This results in the disappearance of elastic tissues in vessels and the dilatation of spiral arteries, which decreases the peripheral vascular resistance of the placental bed. Thus, normal growth of the placenta and fetus can be ensured by smooth influx of more blood into the placental intervillous space. These changes are normally completed in the first 6-7 weeks of pregnancy (Demir et al., 2007). Thus, we studied embryonic villi at about 7 weeks of pregnancy since this is the point during early pregnancy when the remodeling of the decidual spiral arteries is nearly completed by the trophoblast cells.

Studies (Rimm et al., 2009; Klatsky et al., 2010; Calhoun et al., 2011; Tsoumpou et al., 2011; Sazonova et al., 2012) showed the function of trophoblast cells in early pregnancy after IVF-ET differs from that through natural pregnancy in terms of antioxidant capacity, growth, proliferation, invasion, apoptosis, and vascular development, and the pregnancy outcome depends on the self-healing balance of trophoblast cells. We chose some key factors for trophoblast cell invasion and placenta forming, and compared the proteomic expression between natural conceived pregnancy and IVF-ET pregnancy. The expression of EGFR, tubulin-α, Bcl-2, and metallothionein in the placental villi in the IVF-ET group was significantly lower than that in the control group (P<0.05); however, AFP expression was significantly higher in IVF-ET group than in control group (P<0.05)

Epidermal growth factor (EGF) is an important growth factor that binds to EGFR on trophoblast cells, stimulates the receptor tyrosine kinases, and triggers their own and substrate phosphorylation reactions, thereby increasing the nutritional intake by villi, promoting proliferation and differentiation of the placenta, promoting trophoblastic cell survival, and increasing the secretion of placenta (Schwenke et al., 2013). Fu et al.'s study showed (Fu et al., 2010) that EGF promotes trophoblast cell growth in a dose-dependent manner, and EGFR deficiency results in reduced trophoblast proliferation. Fu et al also found EGFR with enzyme activity increased significantly during the period of placenta formation, but with the progress of pregnancy, the expression of EGFR decreased, although in IVF-ET cases the expression of EGFR increased significantly in the late pregnancy. The results of the present study showed that the expression of EGFR in the villous tissue in IVF-ET group was significantly lower than in the control group. We speculate that in full term delivery cases, the expression of EGFR was compensated in middle and late pregnancy.

Tubulin-α is a microtubule structural protein, and Johnstone's research (Johnstone et al., 2011) proved that its expression is significantly lower in the placenta of women with gestational hypertension, which is associated with intrauterine hypoxia and trophoblast cell apoptosis due to hypoxia. Therefore, in addition to maintaining the normal structure of trophoblast cells, the function of tubulin-α in these cells is mainly to act as an antioxidant in early villi. In our present study, the expression of tubulin-α in the villi tissue of women in IVF-ET group was significantly lower than that in control group, which is in agreement with the results of previous studies. Thus, we consider that *in vitro* manipulation of IVF-ET procedure may affect cell microstructural proteins development.

The main role of Bcl-2 is to inhibit cell apoptosis. In normal tissues, the expression of the Bcl-2 protein plays a key role in balancing cell growth and apoptosis. High expression of Bcl-2 protein can inhibit apoptosis and plays an important role in the maintenance of pregnancy. Lodhi (2013) recently found that Bcl-2 is expressed in

syncytiotrophoblasts of villus and cytoplasm and membrane of decidual cell during early pregnancy, and it is weakly expressed in syncytio-trophoblasts. In our present study, the expression of Bcl-2 in villous syncytiotrophoblasts is lower in pregnancies conceived by IVF-ET, which may increase apoptosis occurrence.

Metallothionein is mainly located in the cytotrophoblasts, decidual cells, and small vascular endothelial cells of the placenta. Metallothionein expression in the placenta is important to the structural integrity and function of the placenta. A study (McAleer and Tuan, 2001) of an *in vitro* trophoblastic cell line, JEG-3, suggested that although metallothionein does not ameliorate oxidative stress-induced perturbation of some trophoblastic functions, its expression is critical for protecting these cells against severe oxidative stress-induced apoptosis. A Chinese *in vivo* study (Ma et al., 2006) found similar results in terms of the significance of metallothionein expression in the placentas of women exposed to low levels of lead during pregnancy. In the present study, the expression of metallothionein in the villi tissues of women in IVF-ET group was significantly lower than that in the control group.

AFP is mainly synthesized in the fetal liver, and peaks at 30 weeks of pregnancy, after which it gradually decreases, approaching the mean adult level 1 year after birth. For a long time, research on AFP has focused on cancer-related mechanisms. However, in recent years, researchers have found that trophoblast cells' invasion, infiltration, and remodeling of endometrium and spiral arteries during early development are similar to those of tumor cells. The normal physiological role of AFP is unknown but a variety of diverse functions have been postulated, including ligand binding and transport, regulation of growth and the immune system, and a role as an antioxidant, indicating its potential significance for the growth and development of a healthy fetus. Duc-Goiran et al.'s study indicated (Duc-Goiran et al., 2006) that under normal circumstances, the expression of AFP in trophoblast cells is weak, and its expression is mainly located in discontinuous regions, at junctions between two villi and at duding sites. The main role of AFP is to facilitate the development of the placenta and the compensatory mechanism is only expressed when the blood vessels have developed abnormally. Microscopically, pathologic changes have been observed in syncytiotrophoblast cells of anembryonic pregnancies, and AFP is strongly expressed by villous trophoblastic cells compared with in embryonic pregnancies (Aydin et al., 2006). The present study showed the expression of AFP in the villous tissue in IVF-ET group was significantly higher than in the control group. It may also support that after IVF-ET, vascular development of trophoblasts declines, and compensation is required to increase the expression of AFP, so as to ensure normal vascular development in the placenta during the second and third trimesters, as well as to maintain the normal function of the placenta and protect the exchange of substances and nutrients between the mother's womb and the fetus.

As to the clinical results of these patients, the expression of EGFR, tubulin-α, and metallothionein was weaker in the case resulting in miscarriage than in the other six cases resulting in full-term pregnancy, which suggests the vital role of protective factors and the ability of nutrient cells to grow during the first trimester. The staining intensity of Bcl-2, tubulin-α, and metallothionein were very weak in the early preterm labor case, which suggests that apoptosis of nutrient cells increased during the second trimester of pregnancy and exceeded their growth ability, which is one of the reasons for early preterm labor. The case with velamentous cord insertion showed weak tubulin-α staining intensity, which shows that cell structure proteins are important for placenta formation and development during early pregnancy.

Previous research (Norwitz, 2007) suggests that a lack of trophoblast invasion in the first trimester can lead to miscarriage, ectopic pregnancy, placenta previa, gestational hypertension, etc., while excessive trophoblast invasion is likely to cause placenta implantation, hydatidiform mole, or even chorionic carcinoma. Many *in vitro* manipulations are performed on the gametes and embryos during the IVF-ET process, including ovum collection, *in vitro* embryo culture, *in vitro* artificial insemination, embryo freezing and recovery, artificial assisted incubation, and artificial migration; and details such as the *in vitro* operation time and illumination, laboratory environment, air quality, the pH value of nutrient solutions, and the influence of negative pressure on ova during the collection process are likely to affect embryonic development and implantation, which may cause dysfunction of embryonic implantation, thus leading to implant failure or increased long-term obstetric complications. In the present study, six patients underwent fetal reduction after IVF-ET reached full term pregnancy, which suggested that although IVF-ET can change trophoblast cell function in the first trimester, compensatory mechanisms during the second and third trimesters may normalize trophoblast function, thus maintaining normal placental function.

This experiment has some limitations, such as small sample size, small amount of villi tissue after aspiration in fetal reduction restricting for quantitative research, and additional studies are warranted to address these. Moreover, further exploration of the compensatory mechanism is necessary to provide the theoretical basis and experimental data for improving the pregnancy rate after transplantation and reducing complications during the perinatal period and birth defect rates after IVF-ET.

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