Changing etiology of tubal pregnancy following IVF

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BACKGROUND: Tubal pregnancy (TP) is twice as common following IVF when compared with natural conception. This is surprising, since embryo transfer is aimed for an accurate area in the uterine cavity. We thus hypothesized that either the embryo or the Fallopian tube actively participates in a pathological process leading to implantation outside the uterine cavity. Since we recently found that E-cadherin expression is a useful marker of endometrial receptivity, we considered that it may have a role in TP following IVF. Therefore, the aim of this study was to compare E-cadherin expression and localization in tubal implantation sites from spontaneous TP and TP post-IVF.

METHODS: We compared E-cadherin immunohistochemistry levels on cross-sections of Fallopian tubes in 11 spontaneous (antegrade) versus 13 post-IVF (retrograde) TP. The intensity of immunoreactivity was scored in a semi-qualitative blinded manner. RESULTS: The semi-quantitative intensity score in IVF tubal samples was more than double that observed in spontaneous TP (16.9 versus 7.3, respectively, \( P < 0.0005 \)). E-cadherin showed the most intense immunostaining in cytotrophoblast cells of chorionic villi in ectopic TP post-IVF compared with negative or weak staining in spontaneous ectopic TP.

CONCLUSIONS: E-cadherin can serve as a marker of implantation. Differential expression of this adhesion molecule in TP post-IVF, when compared with natural conception, may reflect a different mechanism of embryo implantation. Moreover, the observation that E-cadherin is mostly expressed in trophoblasts, and not in the tubal wall, suggests that the preimplantation embryo may actively participate in locating a suitable implantation site.

Keywords: E-cadherin; ectopic pregnancy; IVF; implantation site

Introduction

Most ectopic pregnancies (>96%) appear in the Fallopian tubes and are commonly known as tubal pregnancy (TP), which can occur as reproductive failure (i.e. in women not using contraceptives) or as contraceptive failure. The pathophysiology of TP results from a tubal mechanical block to the antegrade progress of the developing embryo. Ectopic pregnancy indeed frequently occurs in women with impaired tubal function (Russell, 1987). The strongest risk factors for TP include surgically visualized tubal pathology originating from pelvic infection, endometriosis or previous surgery (Pisarska et al., 1998). Other risk factors include patient age, smoking and prior TP (Bouyer et al., 2003).

The first IVF treatment resulted in TP (Steptoe and Edwards, 1976) and the reason why this pathology is still more common in IVF is unclear. Since embryo transfer does not directly involve the Fallopian tube, it would be expected that assisted reproductive treatment (ART) might actually reduce the risk of TP. Nevertheless, among women undergoing IVF, 2–5% of resultant pregnancies are tubal (SART/ASRM, 2002, 2004), a rate which is 2–3% higher than that in the general population (Pisarska et al., 1998). However, the IVF rate may actually have decreased somewhat in recent years (SART/ASRM, 2004; Clayton et al., 2006).

As opposed to antegrade tubal blockage as the main cause of TP in a spontaneous pregnancy, the reason that IVF embryos migrate from the uterine cavity only to implant in the Fallopian tube is not so clear. The most likely cause is direct extrusion of embryos through the tubal ostia by the hydrostatic pressure associated with embryo transfer (Bearman et al., 1986). Retrograde embryo migration into diseased tubes is believed to be the main cause of TP following IVF.

Early diagnosis is essential for the prevention of significant maternal morbidity and mortality. Increased risks may arise from the effect of medications for ovulation induction through hormonal fluctuations on tubal function (Fernandez et al., 1991). Factors that could triple the risk for TP following IVF include tubal disease, endometriosis, smoking and age (Fernandez and Gervaise, 2004). The aberrant embryo–implantation into the Fallopian tube in IVF-embryo transfer could be viewed as a subclass of failed in utero implantation.
The role of the uterus in the process of conception is limited to a critical period termed the ‘window of implantation’ spanning between Day 20 and Day 24 of a regular menstrual cycle (day LH+7 to LH+11) (Psychoyos, 1973). During this period, endometrial receptivity enables the apposition, adhesion and invasion of the embryo into the uterine wall. In recent years, there has been an effort to identify the participants in this process. Progesterone stimulation of an estrogenized endometrium leads to the production of proteins and adhesion molecules (e.g. integrins and cadherins) and pinopods, which are considered to be associated with the implantation window. Conditions negatively affecting embryo implantation in utero manifest as decreased levels of endometrial receptivity markers (Meyer et al., 1997; Lessey, 2002). We recently reviewed the role of various molecular changes, which form the basis of the embryo implantation process, including cytokines, lipids and cell adhesion molecules (CAMs) (Achache and Revel, 2006). CAMs crucial for initial attachment between the embryo and the endometrium include integrins (Lessey, 1997), L-Selectin (Genbacev et al., 2003), immunoglobulins (Thomson et al., 1999) and cadherins (Achache and Revel, 2006).

E-cadherin is a transmembrane glycoprotein localized at the adherens junction, which are specialized regions on the lateral side of the epithelial plasma membrane. In the adherens junction, E-cadherin is connected to the intracellular actin filament-attachment proteins (α- and β-catenin, vinculin and actinin). E-cadherin is expressed by a variety of tissues and plays a central role in embryogenesis, gastrulation, neurolation and organogenesis.

The localization of E-cadherin in human trophoblastic cells, as well as its expression in luminal and glandular endometrial epithelium throughout the menstrual cycle, supports the involvement of this molecule in the embryo implantation process. E-cadherin is presumed to be essential for embryo development and blastocyst implantation.

The aim of this study was to evaluate E-cadherin localization at the implantation site in TP. Using an immunohistochemical study, we semi-quantitatively compared E-cadherin level in spontaneous (antegrade) versus post-IVF (retrograde) TP. Our hypothesis was that IVF causes abnormal E-cadherin over-expression in some areas of the Fallopian tube. This would then coaxes the blastocyst from the uterine cavity, where it had been carefully set by an embryo transfer catheter, resulting in embryo implantation at the ectopic site.

Materials and Methods

Patients

We included patients who underwent salpingectomy due to TP at the Hadassah hospital during the years 1998–2006. The research group (n = 13) consisted of patients who had TP following IVF, whereas the control group (n = 11) consisted of patients who had a spontaneous TP. Medical files, hysterosalpingograms and previous diagnostic laparoscopy reports were analyzed. Patients with a known previous tubal pathology, history of pelvic inflammatory disease (PID) or endometriosis were excluded.

Tissue preparation

Cross-sections of formalin-fixed, paraffin-embedded Fallopian tubes removed by laparoscopic salpingectomy for TP were selected from the archives of the Department of Pathology Hadassah Medical Center and re-examined. Blocks of selected cases which included the implantation site were re-cut at 4 μm onto double-frosted slides. Pathological revision (by D.P.) of slides prior to inclusion and staining confirmed the diagnosis and the site of tubal implantation and excluded other pathology in those tubes.

Immunohistochemistry

Formalin-fixed paraffin-embedded sections were deparaffinized in xylene twice for 5 min and rehydrated in 100% ethanol twice for 3 min, followed by 95% ethanol for 1 min and 80% ethanol solution for 1 min. Antigen retrieval was achieved by microwave heating for 20 min in target retrieval solution (Dako REAL™ Dakocytomation, Glostrup, Denmark). The specimens were allowed to cool for 20 min and washed twice in Tris buffered saline pH 7.4 (20 mM TBS, 10 mM CaCl₂, 1% BSA). Endogenous peroxidase was blocked using 0.03% hydrogen peroxide [EnVision® system-HRP (diaminobenzimide, DAB), Dakocytomation. Glostrup, Denmark] for 5 min and the specimens were then rinsed in TBS. Blocking was carried out with 2% normal goat serum (Sigma Chemical Company, St Louis, MO, USA) for 30 min at room temperature. The sections were incubated overnight with an E-cadherin mouse monoclonal antibody (1:500 dilution, HEC-1 clone, Alexis Biochemicals, Lausanne, Switzerland) at 4°C. The specimens were then washed in two changes of TBS prior to incubation for 45 min at room temperature with HRP labeled polymer conjugated to goat anti-mouse immunoglobulin (EnVision® kit). The slides were subsequently washed twice in TBS and incubated for 10 min in DAB and Chromogen solution (EnVision® kit). The slides were counterstained using Meyer’s hematoxylin solution (Sigma Chemical Company) for 4 s and then washed in water for 2 min. Slides were mounted for microscopic examination.

Assessment of E-cadherin expression

The study included a semi-quantitative analysis of E-cadherin staining on slides from spontaneous and post-IVF TP. Semi-quantitative analysis has been demonstrated to be a reliable assessment (Cross, 2001). The pattern and level of E-cadherin staining was assessed by an experienced pathologist (D.P.) who was blinded to the origin of each slide. We excluded slides with <10% staining in the examined area. Since distribution of the staining was focal in all cases, the areas with the most intense staining on each slide were chosen for scoring. We used a semi-quantitative scoring system that took into account the number of E-cadherin immunoreactive cells and the staining intensity level (level 0 for no stain, level 1 for weak stain and level 3 for strong stain). E-cadherin immunostaining was evaluated separately for the free floating villi and the anchoring villi with the implantation site.

Statistical analysis

The Mann–Whitney test was used to determine the significance of differences in the average staining intensity between the two groups. The demographic data of the related groups were also analyzed by Mann–Whitney test, Student’s t-test and χ² test.

Results

We compared 13 cases of IVF-TP to 11 cases of spontaneous TP. The mean ages of the IVF-TP patients and the spontaneous
TP patients were similar (Table I). As expected, however, the IVF patients had significantly lower parity than the spontaneous TP patients (0.69 ± 0.9 versus 2.36 ± 2.6, respectively; \(P = 0.047\)). We also noted that EP was diagnosed earlier in IVF patients when compared with spontaneous TP patients (41.6 ± 10.8 versus 53.3 ± 9.2 days from last menstrual period (LMP), respectively; \(P = 0.014\)). Earlier diagnosis of TP in IVF pregnancies is probably the result of the close follow-up of IVF patients.

The semi-quantitative intensity score in IVF tubal samples was more than double that observed in spontaneous TP (16.9 versus 7.3, respectively; \(P = 0.0004\)) as shown in Fig. 1. All scores were blindly measured on slides depicting only the TP implantation sites. The placental bed was seen in the wall of the Fallopian tube. Extra-villous trophoblastic cells were seen infiltrating it. Strong positive immunostaining for E-cadherin was seen in the placental bed in post IVF-TP (Fig. 2A) whereas weak or negative staining was observed in spontaneous TP (Fig. 2B). Strong immunostaining for E-cadherin was seen in cytотrophoblasts cells (arrows) and intermediate trophoblasts cells (asterisk) of the chorionic villi in post-IVF ectopic TP (Fig. 2C), whereas negative or weak staining was observed in spontaneous ectopic TP (Fig. 2D). There was no difference in the pattern nor intensity of immunostaining of the tubal epithelium between the two groups.

### Discussion

The reason why the rate of EP following IVF-embryo transfer is 3–5-fold higher (Marcus and Brinsden, 1995) than that in the general population is still unknown. The general explanation attributes tubal dysfunction to an hormonal effect

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**Table I.** Demographic and medical data of post-IVF and spontaneous TP patients.

<table>
<thead>
<tr>
<th></th>
<th>IVF</th>
<th>Spontaneous</th>
<th>(P)-value</th>
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<tbody>
<tr>
<td>Patient age(^a)</td>
<td>31.69±4.4</td>
<td>30.82±5.7</td>
<td>0.678</td>
</tr>
<tr>
<td>Gravidity(^b)</td>
<td>2.46±1.45</td>
<td>4.27±2.76</td>
<td>0.072</td>
</tr>
<tr>
<td>Parity(^b)</td>
<td>0.69±0.9</td>
<td>2.36±2.6</td>
<td>0.047</td>
</tr>
<tr>
<td>Previous Cesarean section(^c)</td>
<td>7.7%(1/13)</td>
<td>18.2%(2/11)</td>
<td>0.58</td>
</tr>
<tr>
<td>Previous spontaneous abortion(^b)</td>
<td>0.77±1.1</td>
<td>0.91±1.04</td>
<td>0.649</td>
</tr>
<tr>
<td>Previous ectopic pregnancy(^c)</td>
<td>23.1%(3/13)</td>
<td>0%(0/11)</td>
<td>0.22</td>
</tr>
<tr>
<td>Mean hCG level at laparoscopy(^a)</td>
<td>6664±3553</td>
<td>3862±3995</td>
<td>0.13</td>
</tr>
<tr>
<td>Mean pregnancy age(days)(^a)</td>
<td>41.58±10.8</td>
<td>53.3±9.23</td>
<td>0.014</td>
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\(^a\)\(t\)-test.

\(^b\)Mann–Whitney.

\(^c\)Chi-square test.

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**Figure 1:** E-cadherin levels in the placental bed in spontaneous versus post-IVF tubal pregnancy. Immunoreactivity for E-cadherin was significantly different between the two groups: the intensity of immunostaining in the post-IVF TP group was stronger (16.88) compared with the spontaneous TP group (7.32, \(P = 0.0004\)). No significant differences between the groups were established for the epithelial tubal cells outside the implantation zone.

**Figure 2:** Expression of E-cadherin in ectopic tubal pregnancy. Extravillous trophoblastic cells in the infiltrating placental bed. Positive immunostaining for E-cadherin in ectopic tubal pregnancy post IVF (A) versus negative staining in spontaneous tubal pregnancy (B). Immunoreactivity for E-cadherin in cytотrophoblasts cells (arrows) and intermediate trophoblasts cells (asterisk) of chorionic villi in TP post-IVF (C) versus negative staining in spontaneous TP pregnancy (D).
(Fernandez et al., 1991), but this does not explain the absence of normal implantation in utero. Another proposal is that embryos are sometimes transferred too deeply into the uterine cavity (Pope et al., 2004). Tubal damage among women with ectopic pregnancies who had used IVF because of endometriosis or unexplained infertility has also been reported (Dubuisson et al., 1991).

The hypothesis of our study was that a different presentation of E-cadherin, a potent adhesion molecule, at the tubal embryo implantation site could reveal a pattern for TP in IVF-embryo transfer. Indeed we describe in this manuscript, for the first time, an intrinsic difference between TP following spontaneous conception compared with that following IVF. E-cadherin is strongly expressed at the tubal embryo implantation site only in patients following IVF. We thus suggest that biological, rather than mechanical factors, give rise to TP post-IVF as opposed to spontaneous TP which probably involves a mechanical obstruction to the advancement of the developing blastocyst into the uterine cavity.

E-cadherin has been characterized as being involved in anchoring placental villi and is down-regulated following extravillous differentiation (Babawale et al., 1996; Zhou et al., 1997a,b). Abnormal persistence of E-cadherin has been reported in pre-eclamptic and molar pregnancies (Shih and Kurman, 1997, 1998; Zhou et al., 1997a,b). Floridon et al. (2000) reported the localization of E-cadherin positive staining in placental tissue of ectopic pregnancies. They elegantly demonstrated that villous trophoblastic expression did not differ between normal, ectopic or molar pregnancies. The staining was positive in cytotrophoblasts and intermediate trophoblasts, but negative in syncytiotrophoblasts. Similar to our findings, the staining was clearly localized; the maternal stromal cells of the Fallopian wall were all E-cadherin negative. In their study (Floridon et al., 2000), the presence of E-cadherin was demonstrated throughout the first, second and third trimesters, thus the E-cadherin level should not be affected in our study, by the 10 day discrepancy between the research and the control groups.

Evidence from E-cadherin knock-out mice demonstrates that E-cadherin is essential for normal implantation. E-cadherin homozygous −/− were unable to form functional trophoderm and died around the time of implantation (Larue et al., 1994; Riethmacher et al., 1995). Interestingly, cadherins demonstrate slightly different patterns between in vivo and in vitro culture preimplantation mouse embryos (Wang et al., 2005). E-cadherins have also been shown to be involved in the menstrual cycle in endometrial and peritoneal cells as well as in pathological conditions such as endometriosis (van der Linden et al., 1994).

In our study, strong staining for E-cadherin in the Fallopian tubes of IVF patients is localized to trophoblastic cells. This may indicate that the relatively higher risk for TP following IVF-embryo transfer is primarily embryonic and not tubal in origin. The implantation site is thus affected by the preimplantation embryo quality. Thus, TP in IVF patients with normal Fallopian tubes may be linked to embryos with lower implantation capability. Morphological quality of the IVF embryos was, however, normal with means of 4-cell grade B–C and 6-cell grade embryos transferred on Days 2 and 3, respectively.

Preimplantation embryos in vivo develop in an undefined complex milieu containing nutrients, ions and macromolecules, many of which may be used to satisfy metabolic requirements (Leese, 1995). The culture systems used for IVF are somewhat different from the natural environment conditions in vivo, and this may affect the metabolism and development of the preimplantation embryo (Houghton and Leese, 2004). In addition, the preimplantation embryo is regulated in vivo by endocrine, autocrine and paracrine factors that are differently expressed in the process of IVF. These differences must have some influence on the preimplantation embryo. Over-expression of E-cadherin may be a reflection of these differences.

Discordance between endometrial and embryonic adhesion molecules may prevent embryonic intrauterine adhesion. The IVF embryos may be ‘non sticky’ at the time of embryo transfer and later wander into the Fallopian tube. By this time, adhesion molecules may have finally presented themselves on the trophoblasts, resulting in aberrant implantation. Thus, TP may result from a delay, and not an absence, in the presentation of adhesion molecules.

The concept of delayed implantation following IVF is not new. Indeed, serum beta HCG levels, measured subsequent to IVF-embryo transfer, increase at an ordinary rate, but are 1.5 days later than in spontaneous conception. This phenomenon results from a delay in implantation (Korhonen et al., 1996). Delayed implantation, typical of ectopic pregnancy after IVF-embryo transfer, represents the postponed embryo entrance into the Fallopian tube.

Embryos, inserted into the uterine cavity following a few days of culture, are of a wide range of developmental quality. Delayed secretion of adhesion molecules, by poor quality embryos, produces a lower chance of intrauterine pregnancy and a higher risk of TP. Indeed, data from 2009 ectopic pregnancies find low embryo quality to be an independent risk factor for TP (Clayton et al., 2006). In contrast, when high quality embryos were transferred, the odds ratio for TP dropped by one-third. The risk of TP following oocyte donation, when very high quality embryos from young donors are transferred, is low and compares with that after spontaneous conception (Clayton et al., 2006).

In conclusion, we are suggesting the following model. The embryo, recently transferred to the uterine cavity, rolls on the endometrium in search of the optimal area for implantation (Achache and Revel, 2006). The embryo and the endometrium both secrete adhesion molecules that enable the embryo to achieve apposition, adhesion and penetration (Norwitz et al., 2001) at optimal temporal and spatial conditions. When this process is faulty, TP or failed implantation could occur.

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References


