Abstract

Objective: It is well accepted that hatching blastocysts have the highest implantation potential provided that inner cell mass (ICM) and trophectoderm (TE) have formed adequately. Though, there are a wide variety of possible hatching spots around the blastocyst surface, no data exist analyzing the actual influence of this site on implantation behavior. This semi-prospective study was set up in order to evaluate if blastocysts implant at a higher rate, if the opening of the zona pellucida is located close to the inner cell mass ICM, which corresponds to the area that will later drive invasion into the endometrium (syncytiotrophoblast).

Materials and Methods: During the initial phases of the study images of all transferred hatching blastocysts were checked if they herniated near the ICM (study group) or TE (control group). As soon as it became evident that blastocysts hatching close to the embryoblast were superior in terms of outcome they were selected prospectively.

Results: A total of 108 patients were involved, 82 of them had a homogeneous transfer in terms of the hatching site. Patients from the study group showed a trend towards a higher (p = 0.06) clinical pregnancy rate (72.4%) as compared to the control group (50.9%). Implantation rate, however, was significantly increased (66.7% vs. 40.8%) (p = 0.009).

Discussion: Theoretically, hatching at the embryonic pole could accelerate contact between those trophectodermal cells supposed to draw the blastocyst into the uterine wall and the endometrium. This mutual interaction between blastocyst and uterus may be hindered or delayed if hatching takes place opposite the ICM and/or if hatching difficulties occur.

Keywords: blastocyst, hatching, inner cell mass, trophectoderm

Özet

Hücre Fitikaflman›n Konumu Zona Pellucidadan Ç›kan Blastokistlerin ‹mplantasyon Davran›fllar›n› Öngörmektedir

Amaç: Zona pellucidadan çiçen blastokistlerin, iç hücre kütlesinin (ICM: inner cell mass) ve trofoektodermi (TE) uygun gelişmiş olması ön Burtonlu, en yüksek implantasyon potansiyeline sahip oldukları kabul edilmiş bir gerçektir. Blastokist çevresindeki zona pellucidadan oluşan çiçen noktalarının çikanluğuna rağmen, bu bölgenin implantasyon davranışları türlerindeki gerçek etkisini icoleleyen herhangi bir veri yoktur. Bu, kısmen izlenmemi olan araştırmaların yanıltıcı amaç zona pellucidadaki deligin iç hücre kütlesine (ICM), ki bu sonradan endometriyuma (sinisyyotrofoblast) işlenir soğulayacak bölgeye denk gelir, yakın konumlandirilmiş durumunda blastokistlerin daha yüksek oranda implantle olup olmalarını değerlendirmektedir.

Materyal ve Metot: Araştırma başlangıç sahalarında transfer edilmiş tüm blastokistlerin görüntülerini iç hücre kütlesinin mi (çalışma grubu) yoksa TE’nin mi yakınında (kontrol grubu) oluşturdukları/fitiklafları tür gözden geçirilmiştir. Embriyolastı yakını yerleri zona pelucidayı delen blastokistlerin sonuçlarının daha üstün olduğunu anlaflmasıyla bunlar ileride döntük kullanılmaya amaçla seçilmiştir.

Sonuçlar: Toplam 108 hastanın dahi olduğu araştırmada, 82’sine zona pellucidayı delme bölgesi bakımından homojen transferler yapılmıştır. Çalışma grubundaki hastalar kontrol grubundakilerle karşılaştırıldığında (%50.9) daha yüksek bir klinik ha- milelik oranı (%72.4) eştini sergilemişlerdir (p=0.06). Ayrıca, implantasyon oranı önemli ölçüde artılmıştır (%66.7’ye kars- ı %40.8) (p=0.009).

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Introduction

In IVF laboratories, various preventive strategies have been applied in order to reduce multiple gestations without lowering success rates. One of these non-invasive approaches is to focus on the morphological appearance of oocytes/embryos during the first five days of preimplantation development (1,2). Thus, positive and negative predictors evaluated at different stages may be weighed against each other in order to select the optimal candidates for transfer.

It is a matter of fact that the number of predictive variables increases with the time of in vitro culture. This may, at least in part, explain the reported superiority of blastocyst transfer (BT) over cleavage stage transfer (3,4), though this statement has not remained unchallenged (5-7). However, decision making in terms of transfer is likely to be more promising on the inner cell mass (ICM) and the trophectoderm (TE).

Indeed, blastocyst transfer, especially single blastocyst transfer, has been shown to be an effective method of eliminating multiple births while maintaining high pregnancy rates (8-10). Thus, single blastocyst transfer should be obligatory, at least in patients with good prognosis.

One such positive predictor would be the availability of hatching blastocysts for transfer (11). Expanded blastocysts that started to escape from the zona pellucida (ZP) were shown to achieve higher pregnancy rates (57.7%) as compared to non-hatching (27.8%) ones (12). This may be due to the fact that in this group obviously no hatching difficulties could have occurred. By removing the whole zona pellucida hatching difficulties can be overcome (13) which may result in increased rates of pregnancy and implantation (14,15).

Though there is a wide variety of possible hatching spots around the blastocysts surface, no data exists on the actual influence of this site on implantation behaviour. This semi-prospective study was set up in order to evaluate if blastocysts implant at a higher rate, if the opening of the zona pellucida is located close to the inner cell mass, which corresponds to the area that will later drive invasion into the endometrium (syncytiotrophoblast).

Materials and Methods

During the study period of almost 2 years (January 2005- November 2006) a total of 1184 patients were referred to our IVF clinic. Amongst female indications for assisted re-productive technologies PCO syndrome (2%), endometriosis (5.4%) and tubal sterility (16.8%) were the most frequent ones. However, approximately half of the couples (48.7%) suffered from male factor infertility (n=577). Every fourth couple (23.7%) showed both male and female subfertility (n=281).

According to this distribution, ICSI was planned (n=919) in the vast majority of cases (77.6%), whereas IVF appeared appropriate in 22.4% (n=265) of the cycles.

For controlled ovarian hyperstimulation (COH) two major stimulation regimens were used during the study, a long protocol (25.9%) and an antagonist protocol (72.9%). In the remaining 14 cycles (1.2%) oocyte collection was performed in a spontaneous cycle. In the long protocol, stimulation was initiated with human menopausal gonadotrophin (HMG, Menogon®, Ferring, Kiel, Germany) after down-regulation of the pituitary with the GnRH agonist buserelin (Suprecur®, Aventis Pharma, Vienna, Austria). In the GnRH-antagonist protocol, HMG (Menogon®, Ferring, Kiel, Germany) was started on day 2 of the cycle. In addition, a GnRH-antagonist (Orgalutran®, Organon, Vienna, Austria) was administered after 5-6 days of stimulation, depending on the presence of a 12-13 mm follicle in the ultrasound scan. In all patients ovulation was induced with 5000-10 000 IU human chorionic gonadotrophin (hCG, Pregnyl®, Organon, Vienna, Austria). Routinely, oocyte retrieval was carried out transvaginally under ultrasound guidance 36 hours after hCG administration.

For conventional IVF, oocyte-cumulus complexes were incubated for 3 hours in BM1 medium (NMS Bio-Medical, Praroman, Switzerland) before being inseminated. In preparation for insemination ejaculate of normozoospermic patients was incubated in a Zech glass capillary dish (Astro Med Tec, Salzburg, Austria) which localizes an adequate number of motile sperm without exposure to centrifugation stress (16). In contrast to IVF, spermatozoa for ICSI were separated from the ejaculate by using a swim-up technique. Intracytoplasmic sperm injection was done as previously published (17).

Some 10% of all cycles did not have any embryo transfer due to fact that either no oocytes were collected (n=17) or failed fertilization or embryo arrest was observed (n=102).

However, 1065 women had at least one embryo or blastocyst transferred. According to the guidelines recommended by Racowsky et al. (18) the number of eight-cell embryos on day 3 became a key determinant in our laboratory for selec-
ting conception for embryo or blastocyst transfer. It is important to note that, in this regard, we considered the number of blastomeres to be of more predictive power than the degree of fragmentation (19,20). Thus, less than one third of all transfers (n=310) were done at blastocyst stage (day 5), whereas all other transfers (n=755) were performed at cleavage stage (day 3).

At day 3, routine evaluation of the embryos included number and shape of blastomeres, percentage of fragmentation and presence of multinucleation. At blastocyst stage, however, scoring was more detailed, e.g. an accurate analysis of both blastocyst extension, as well as the morphology of inner cell mass and trophectoderm was performed (11). In particular, size and shape of the inner cell mass was taken into consideration while deciding which blastocysts to transfer (21). These morphological criteria of day 5 are routinely applied in order to select blastocysts for transfer. However, this is the first attempt to further identify those blastocysts with best prognosis, e.g. expanded blastocysts of optimal quality (exclusively grade V according to Gardner and Schoolcraft) (11) that already started to hatch from the zona pellucida, according to the actual location of the herniation (close to embryonic pole or at the opposite region).

Therefore, we analyzed the stored images of all good quality blastocysts that had started to hatch when transferred (11). From the moment it became evident (after 2-3 months of the study period) that blastocysts hatching near the inner cell mass (Figure 1) show better implantation behaviour, we preferentially transferred such blastocysts. In order not to miss any hatching blastocysts all expanded blastocysts were carefully turned around using the holding pipette. This helped a lot in adequately allocating blastocysts to the study group (n=28) (all transferred blastocysts hatching close to the inner cell mass) or the control group (n=48) (hatching with the mural trophectoderm, Figure 2). Of course, some patients (n=26) had inhomogeneous transfers in terms of hatching spot (transfer of two blastocysts with different hatching spots).

Table 1. Implantation behaviour of blastocysts hatching at different spots around the zona pellucida

<table>
<thead>
<tr>
<th></th>
<th>Study group hatching from ICM</th>
<th>Mixed group</th>
<th>Control group hatching from TE</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>29</td>
<td>26</td>
<td>53</td>
</tr>
<tr>
<td>Clinical PR</td>
<td>21 (72.4)</td>
<td>16 (61.5)</td>
<td>27 (50.9)</td>
</tr>
<tr>
<td>MPR</td>
<td>5 (23.8)</td>
<td>5 (19.2)</td>
<td>4 (14.8)</td>
</tr>
<tr>
<td>IR</td>
<td>26/39 (66.7)</td>
<td>21/52 (40.4)</td>
<td>31/76 (40.8)</td>
</tr>
</tbody>
</table>

\(a p=0.009; b p<0.01\)

Values in parentheses are percentages. Mixed group had two blastocyst with different hatching spots transferred.

ICM: inner cell mass; IR: implantation rate; MPR: multiple pregnancy rate; n: number of patients; PR: pregnancy rate; TE: trophectoderm

Statistical comparison was performed using \(¥^2\)- and \(t\)-test. Statistical significance was defined as \(p<0.05\).

Results

During the study period, 108 patients had at least one grade V blastocyst (already hatching) available, which corresponds to 10% (108/1065) of all women who actually had a transfer. Regarding the method of fertilization, 8% of the IVF patients and 11% of the ICSI patients showed hatching blastocysts (\(p=0.2\)); thus, male factor infertility had no influence on blastocyst development, at least in our study. The same was found in terms of controlled ovarian hyperstimulation, since comparable percentages of patients with long protocol (12.5%) and antagonist protocol (9.5%) showed at least one hatching blastocyst in culture (\(p=0.16\)).

Blastocyst transfer during the study period gave a significantly better clinical pregnancy rate (39.4%) as compared to cleavage stage (30.3%) transfer (\(p<0.001\)). The same significant (\(p<0.001\)) difference was observed in terms of implantation rate. In detail, only every fifth embryo transferred on day 3 (22.7%) implanted, whereas the chance for implantation was almost double for blastocysts (39.7%).

However, within the selected group of blastocyst transfers those exclusively consisting of hatching blastocysts had the best prognosis in terms of clinical pregnancy rate (60.2%) and implantation rate (46.7%). Of all hatching blastocysts retransferred 38.9% (65/167) hatched close to the inner cell mass and 61.1% from the other hemisphere. Table 1 indicates that those blastocysts hatching from the embryonic pole implanted at a significantly higher rate (\(p<0.01\)) as compared to those hatching from the mural trophectoderm. In addition, there was a trend (\(p=0.06\)) towards a higher clinical pregnancy rate in the study cohort as compared to the control group.

Discussion

In the developing follicle the cleft between the oocyte and surrounding somatic cells is filled with a glycoprotein layer (15-20 μm), called zona pellucida. This matrix is synthesized.
by the gamete in a coordinate manner (22) and is composed of up to four zona proteins that form filaments of repeating units (zona proteins 2 and 3) cross-linked by zona protein 1 (23-25), thus, ensuring the structural integrity of the zona.

During preimplantation development several functions have been attributed to the zona including species-specific sperm binding, inducing acrosome reaction in order to prevent polyspermy, protecting the integrity of the developing embryo and assisting its oviductal transport. After compaction when cell junctions have already formed zona pellucida has served its purpose and the blastocyst is forced to escape from its protective shell in order not to face necrosis.

While in vivo uterine influence on hatching behaviour may not be denied (26), in vitro spontaneous hatching of the human embryo is supported by the tremendous increase of internal pressure caused by a gradual accumulation of blastocoelic fluid and cellular (mostly trophectodermal) proliferation. At the beginning of the hatching process small vesicles protrude through the zona pellucida. It is important to note that this blebbing does not necessarily indicate the precise location of subsequent hatching (27). However, once a small opening has been generated the trophectoderm starts to herniate. Governed by trophectodermal projections a larger opening is created by mechanical forces, a process supported by electronmicroscopical findings (28). In detail, a specialized placental-like stroma has been observed within the trophectoderm. These “zona-breaker” cells (29) line both sides of the trophectoderm at potential hatching points. Microvilli at the surface and large bundles of contractile tonofilaments enable these specialists to interact with the zona pellucida, presumably acting like a sphincter. Additional mechanical help may come from the phenomenon of blastocyst “breathing” (27), a sequence of rapid collapses and slow re-expansions are considered to assist final extrusion from the ruptured zona pellucida.

Though, the main driving force during hatching is a mechanical one, cellular ultrastructure of the breaker cells, especially the presence of lysosomes and other secretory vesicles, strongly indicates that biochemical processes are involved as well. This is in line with previous work stating that hatching process may also be mediated by zona lysins (29).

In the human, hatching occurs at various regions. While some authors postulate that blastocysts show hatching sites mainly close to the inner cell mass (27), others present contradicting data, finding that most of the blastocysts hatch from the abembryonic pole (28,30). Considering the proportions within a blastocyst, the chance of blastocysts to hatch from the smaller embryonic site is much lower than the likelihood to herniate near the rather extensive mural trophectoderm. Present data support the latter theory since only some 40% of the hatching blastocysts showed an opening of the zona pellucida close to the inner cell mass.

Hatching at the embryonic pole could accelerate contact between those trophectodermal cells that are supposed to draw the blastocyst into the uterine wall and the endometrium. Taking into consideration laws of physics, it may be hypothesized that within a volume of medium used for transfer, gravity will orient the polar blastocyst with the inner cell mass towards the endometrium because of its cellular aggregation. This mutual interaction between blastocyst and uterus may be impaired or delayed if herniation takes place opposite the inner cell mass and/or if hatching difficulties occur.

However, to the best of our knowledge, this semi-prospective study is the first to analyze the impact of different hatching sites on further implantation behaviour. Interestingly, a significantly higher implantation rate could be achieved if blastocysts were transferred that hatched close to the embryoblast (p<0.01). It seems that, in contrast to mouse blastocysts (26), human counterparts have a developmental benefit if they hatch adjacent to the inner cell mass, since this area corresponds to the cells (syncytiotrophoblast) that will later drive invasion into the endometrium.

References


Figure 2. Blastocyst hatching opposite the inner cell mass.


