Journal für

Reproduktionsmedizin und Endokrinologie

– Journal of Reproductive Medicine and Endocrinology –

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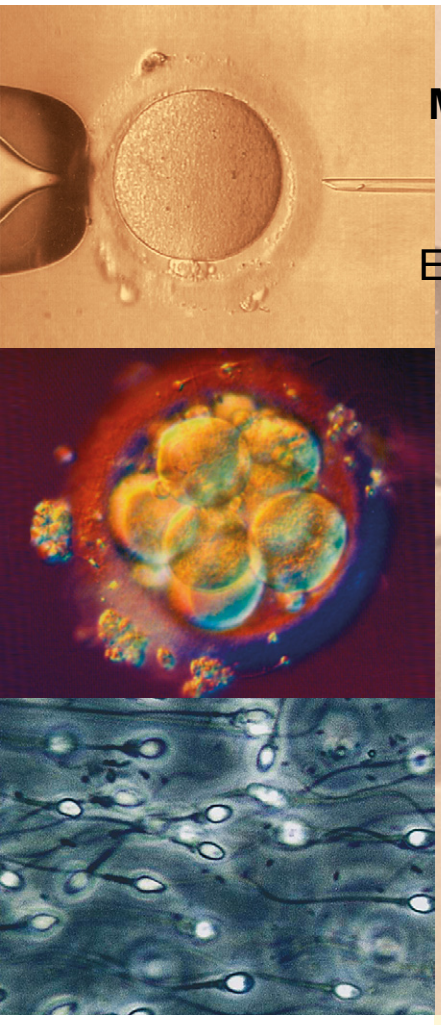

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J. Reproduktionsmed. Endokrinol 2011; 8 (1), 13-20

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Indexed in EMBASE/Excerpta Medica/Scopus

Krause & Pachernegg GmbH, Verlag für Medizin und Wirtschaft, A-3003 Gablitz

Morphological Aspects of Human Blastocysts and the Impact of Vitrification

T. Ebner[1], P. Vanderzwalmen[2], O. Shebl[1], R. B. Mayer[1], M. Moser[1], G. Tews[1]

The topic whether blastocyst culture and transfer is a promising tool in IVF laboratories has been discussed controversially. Discrepancies in outcome may be explained by the fact that formation of a blastocyst on day 5 does not automatically correspond to its viability. Adequate morphological scoring at blastocyst stage (quality of inner cell mass and trophectoderm, expansion, screening for anomalies) would definitely help to significantly reduce the number of blastocysts being replaced, which in turn would limit the number of multiple gestations. Such a strategy could automatically increase the number of blastocysts to be frozen for later replacement. Currently, vitrification challenges slow freezing as the cryopreservation method of choice, since it is faster, more cost-effective and yields at least comparable thawing results. With respect to this, four morphological parameters of vitrified/warmed blastocyts are reported (re-expansion, hatching, necrotic foci and cytoplasmic defects) which can successfully be used for selection purposes in frozen blastocyst transfer. To conclude, blastocyst culture, transfer and cryopreservation is certainly a valuable method in the hands of IVF practitioners and has gained acceptance by many programs throughout the world.

Key words: blastocyst quality, hatching,inner cell mass, re-expansion, vitrification

Morphologische Analyse der Blastozyste und Einfluss der Vitrifikation. Die Meinungen, ob eine Blastozystenkultur bzw. ein Blastozystentransfer vielversprechende Methoden der assistierten Reproduktion sind, gehen auseinander. Ein möglicher Grund für diese Diskrepanzen könnte die Tatsache sein, dass die Bildung einer Blastozyste am 5. Entwicklungstag nicht automatisch bedeutet, dass diese auch vital ist. Eine adäquate morphologische Beurteilung im Blastozystenstadium (Qualität von innerer Zellmasse und Trophektoderm, Expansion, Anomalien) würde sicherlich einer Reduktion der Zahl der zu transferierenden Blastozysten förderlich sein und so weiter zur Verringerung der Mehrlingsschwangerschaften beitragen können. Damit einhergehend würde die Anzahl der zu kryokonservierenden Embryonen natürlich steigen. Um eine exakte Prognose hinsichtlich einer zu erwartenden Implantation nach dem Tauen/Erwärmen erstellen zu können, bieten sich 4 morphologische Parameter zur Selektion ehemals kryokonservierter Blastozysten an: Re-Expansion, Hatching, das Vorhandensein nekrotischer Areale sowie zytoplasmatische Defekte. Zusammenfassend lässt sich behaupten, dass bei detaillierter morphologischer Analyse der Blastozyste deren Kultur, Transfer und Kryokonservierung sehr wohl eine Berechtigung auf dem Gebiet der künstlichen Befruchtung hat. **J Reproduktionsmed Endokrinol 2011; 8 (1): 13–20.**

Schlüsselwörter: Blastozystenqualität, Hatching, innere Zellmasse, Re-Expansion, Vitrifikation

Introduction

Compared to the natural cycle, the situation in IVF is different because controlled ovarian hyperstimulation may cause accidental maturation and ovulation of germ cells of reduced developmental potential. In other words, the actual implantation potential may be overestimated though oocyte morphology, fertilization and cleavage rate may appear inconspicuous at first glance. On the other hand, even embryos of worst quality may sometimes turn out to be viable, e.g. develop to healthy babies.

However, taken into consideration that usually routine laboratories neither have the equipment nor the resources to analyze embryo metabolism [1, 2] or cytogenetical constitution, the only approach to reach the ultimate goal in assisted reproduction, namely a healthy singleton delivery, is non-invasive morphological selection at different developmental stages [3, 4].

Theoretically, any prolongation of in vitro culture up to day 4 or day 5 will allow for a more accurate prediction of developmental capacity. On day 4 (90–100 hours past insemination), blastomeres should have formed numerous tight intracellular junctions finally resulting in a compacting or even a morula-stage embryo. This marks the switch from a cell cluster of individual blastomeres to a relatively smooth mass with indistinguishable cell outlines capable of actively regulating its internal environment. On the fifth day of in vitro culture (114–120 hours) preimplantation development should culminate in the formation of the blastocyst. Once fully developed human blastocysts consist of two different cell types: an outer layer of trophectoderm (TE) responsible for the accumulation of fluid in the blastocyst cavity and specialised for implantation and an inner cell mass (ICM) forming all three germ layers of the fetus.

Morphology before Vitrification

Continual improvement in culture media composition resulting in a higher number of available day 5 embryos had 2 major consequences for embryologists. On the one hand, adequate cryopreservation programs for blastocysts had to be established, and, on the other, there was a need for more detailed blastocyst scoring systems in order to filter out those blastocysts which would implant preferentially.

At the beginning of efficient blastocyst grading some twenty years ago particular attention was focused on develop-

Received: July 15, 2010; accepted after revision: January 31, 2011

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mental stage, e.g. blastocyst expansion [5, 6]. A more recent scoring system [7] took additional morphological features into consideration, namely grade of expansion and morphology of ICM and TE. According to the degree of expansion the blastocysts were scored using Roman numbers in ascending order ranging from grade I (blastocyst cavity less than half of the volume of the embryo) to VI (completely hatched blastocyst). Beginning with full blastocyst stage (grade III) additional assessment of ICM and TE was performed (based on cell number and cohesion) in order to predict developmental competence.

Though the Dokras system [5, 6] was shown to be helpful in routine laboratory work [8] the more detailed Gardner approach [7] allows for reducing the number of transferred blastocysts without limiting pregnancy rate [9] and, therefore, gained higher acceptance in IVF laboratories. A recent randomized study compared the two scoring systems [10]. Although similar numbers of blastocysts were transferred in comparable patient cohorts, the Gardner score turned out to be superior to the Dokras score ($p < 0.05$) in terms of implantation (37.6% vs. 25.0%) and multiple pregnancy (38.6% vs. 17.1%). In addition, there was a trend towards higher clinical pregnancy rates ($p = 0.11$) with the Gardner grading system (66.7% vs. 53.0%).

Blastocyst Cell Number

A factor in common to both scoring systems is the emphasis on blastocyst expansion on the day of planned transfer. Developmental stage of the blastocyst on days 5 or 6 may range from a retarded morula to an expanded or even hatching blastocyst stage and most embryologist rely on visual judgement instead measuring blastocyst diameter. Shapiro et al. [11] accurately measured blastocysts prior to transfer and found out that the diameter of a transferred blastocyst was the most significant variable in predicting clinical pregnancy.

In this context a high variability in cell numbers has been observed. The mitotic activity is considered to be a reliable indicator of blastocyst viability and developmental capacity [12], however, in order to count the actual number of nuclei in a blastocyst its cells have to be fixed. Though it has been suggested [13] that indirect assessment of the total cell number (TCN) without destroying the blastocyst is possible under good inverted optics, the vast majority of studies on TCN were performed using stained cells of spare embryos of reduced quality (donated to research), thus, probably not representing the actual cell number of healthy blastocysts. Early work on TCN faced another drawback, namely the inability of simple culture media (e.g. Earle's balanced salt solution, Ham's F10, medium T6) to adequately support human embryo development in vitro. Apart from achieving lower blastocyst formation rates [14] these authors somehow underestimated mitotic potential of in vitro grown blastocysts.

Cell numbers of blastocysts cultured in rather simple media ranged from 42.0 ± 20.3 to 58.3 ± 8.1 on day 5 [15–18]. However, utilizing sequential media, the rate of mitosis was found to be increased, e.g. 63.9 ± 5.3 to 110.5 ± 9.9 cells on day 5 [19], 166.5 ± 16.0 cells on day 6 [20] and as many as 284.0 ± 13.5 cells on day 7 [20]. The question if co-culture with feeder cells might positively influence TCN [18–20] is discussed controversially [17]. However, it can be summarized that a full human blastocyst at day 5 of development should exceed 60 cells and at least have doubled its cell number on day 6.

It appears quite logical that any phenomenon that severely reduces cytoplasmic volume of the embryo could cause a dramatic loss of cells at blastocyst stage if this is reached at all. Indeed published reports describe [21] that blastomere loss following cryopreservation resulted in significantly lower ($p < 0.01$) blastocyst cell numbers on day 6 (45.0 ± 3.7) as compared to blastocysts derived from fully intact cleavage-stage embryos after thawing (58.4 ± 3.4). Extensive fragmentation at earlier stages showed the same detrimental effect on TCN on day 6 [22], e.g. a significant decrease ($p < 0.01$) in cell count from 68.9 ± 5.5 (embryos without fragmentation) to 29.0 ± 3.6 (> 25% fragmentation). Interestingly, for minimal and moderate levels of fragmentation the reduction in cell number was largely confined to the trophectoderm, while a steady number of ICM cells were maintained. This finding suggests a homeostatic regulation of the lineage that gives rise to the fetus [22].

Cell Lineages

Hardy et al. [15] were one of the first to realize certain interesting differences in the growth rate of both cell lineages. In general, mitotic rate of the trophectoderm is approximately 1.5 times higher than that of the ICM; however, compared to some other mammals, the overall proportion of the inner cell mass is relatively high, e.g. 34% of all cells on day 5, 51% on day 6 and 37% on day 7 [15]. The striking peak on day 6, with half of all cells in the blastocyst being part of the ICM, is explained by an increase in ICM growth rate between days 5 and 6, a time when the number of TE cells is virtually unchanged. However, the next day (day 7) the original ratio is re-established since TE cells are shown to double between days 6 and 7, while the mitotic rate of ICM cells remains constant [15]. Since there is widespread cell death of even morphologically normal cells in both cell lineages [15] it is suspected that the maintenance of cell number within the blastocyst cell types is regulated by apoptotic phenomena [23].

Inner Cell Mass

It can be summarized that the health of a blastocyst is strongly dependent on the overall cell number [24] but also on the adequate formation of both cell lineages. This brings us back to the morphological scoring of inner cell mass and trophectoderm [7]. According to this system, the embryoblast is considered to be optimal (grade a) if the ICM showed a tight package of numerous cells (Fig. 1). Any reduction in number and contact affected the quality of this cell lineage, thus, loosely grouped cell accumulations are scored b whereas absence or presence of only few cells randomly distributed within the cavity of the blastocyst are classified as grade c (Fig. 2).

Optimal blastocysts have been defined even more precisely. Quantitative grading of inner cell masses emphasized the importance of ICM size and shape [25]. In contrast to blastocyst expansion and TE cell number, ICM size was significantly related to implantation ($p < 0.006$). Blastocysts showing an ICM of less than 3800 μm^2 showed lower implantation

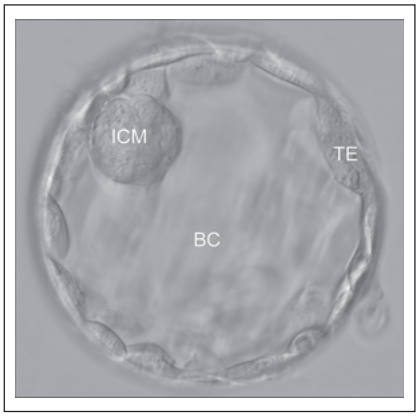

Figure 1: Hatching blastocyst of optimal quality (Vaa) with the hatching site being at the 4 o'clock position. BC: blastocyst cavity; ICM: inner cell mass; TE: trophectoderm

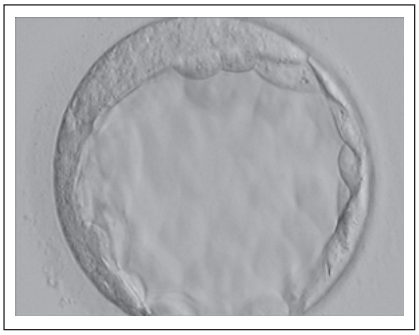

Figure 2: Blastocyst (IVcb) with no visible inner cell mass (comparable to trophoblast vesicle). Trophectoderm quality is reduced since the left hemisphere consists only of a few large cells.

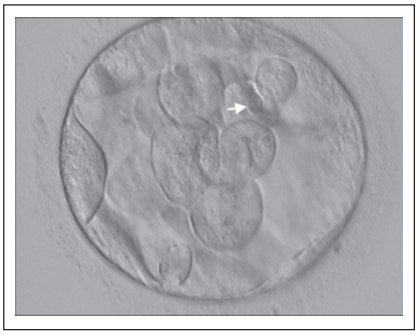

Figure 3: Full blastocyst (IIIbb) showing cell lineages with a limited number of cells. Arrow indicates presence of a necrotic focus in the inner cell mass.

rates (18%) compared to blastocysts with a larger ICM, e.g. > 4500 μm² (45%).

As interesting as these data are, it has to be emphasized that Richter et al. [25] measured expanded blastocysts of different size, e.g. ranging from 155–265 μm. Table 1 (unpublished data) indicates that the size of the embryoblast is closely related to the degree of expansion. This seems to be associated with a more peripheral location of the ICM within the blastocyst cavity as the blastocyst expands and/or an increased cohesion within ICM cells. The latter is further supported by the observation that at full blastocyst stage number of ICM cells can still be estimated (Fig. 3), while at expanded stage its number can not be identified accurately. Taking into consideration these empirical data, it was not surprising that the paper of Richter and co-workers [25] noticed a reduction in ICM size of day 6 blastocysts as compared to day 5 ones (3891 μm² vs 4458 μm²; p = 0.0016).

Present results based on all consecutive single blastocyst transfers of the year 2008 (Tab. 1), however, could only find a significant correlation (p < 0.05) between size of the embryoblast (5413 vs 4141 μm²) and clinical pregnancy at full blastocyst stage (grade III) but not at expanded stage (grade IV and V). However, present data provides first evidence that size of the embryoblast does not affect obstetric and neonatal outcome.

In addition, the above mentioned authors [25] evaluated the possible influence of ICM shape on outcome by introducing the roundness index (RI) which represents the length-to-width proportion of the inner cell mass. In detail, blastocysts with extreme RI values of < 1.04 (almost round) and > 1.20 (too oval) had a worse prognosis (implantation rates of 7% and 33% respectively) compared to those with slightly oval (RI: 1.04–1.20) ICMs (58%). Implantation rates were highest for embryos with both optimal ICM size and shape (71%).

Striving to replace blastocyst with large ICM embryologists should not forget that disproportionately oversized ICMs, e.g. with apoptotic processes not working as they are supposed to [22], could cause problems in maintaining healthy central cells (because of the increased distance over which nutrients and oxygen have to diffuse) and/or could contribute to large-offspring syndrome [26].

Trophectoderm

In a similar way as ICM, Gardner and Schoolcraft [7] classified the TE. The outer layer is considered to be optimal if it consists of numerous sickle-shaped cells forming a cohesive epithelium (grade a). If number and cohesion of these cells is somewhat reduced, i.e. characterized by the presence of several

Table 1: Obstetric and neonatal outcome of pregnancies deriving from single blastocyst transfer of blastocysts of different inner cell mass (ICM) size.

	Grade III		Grade IV		Grade V	
hCG	**pos**	**neg**	**pos**	**neg**	**pos**	**neg**
n	8	14	22	26	27	28
size of ICM (μm²)	5413 ± 393*	4141 ± 1416*	4275 ± 1264	4579 ± 1630	3898 ± 1421	4271 ± 1710
roundness index (RI)	1.38 ± 0.06	1.29 ± 0.20	1.31 ± 0.24	1.36 ± 0.31	1.38 ± 0.31	1.33 ± 0.33
positive β-hCG	36.4%		45.8%		48.2%	
clinical pregnancy rate	36.4%		39.6%		41.1%	
babies born	8		19		24	
gestation week	40.5 ± 0.5		39.0 ± 1.0		39.5 ± 1.8	
weight	3705 ± 617		3222 ± 525		3250 ± 465	
sex ratio (m/f)	1.0		0.39		0.83	
malformations	0		1 (5.6)		1 (4.3)	

* p < 0.05

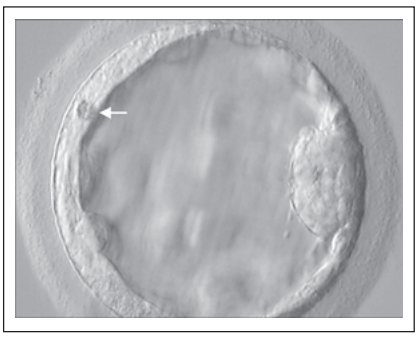

Figure 4: Full blastocyst (IVac) showing rudimentary trophectoderm with necrotic focus (arrow).

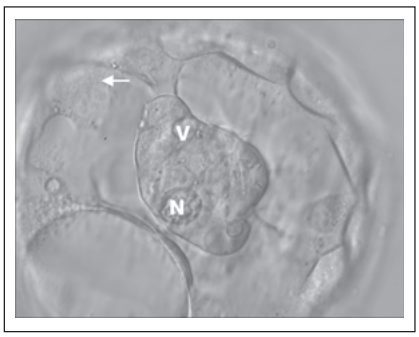

Figure 5: Inner cell mass of optimal size and shape. Reduction in developmental potential is due to the presence of vacuole (V) and necrotic focus (N).

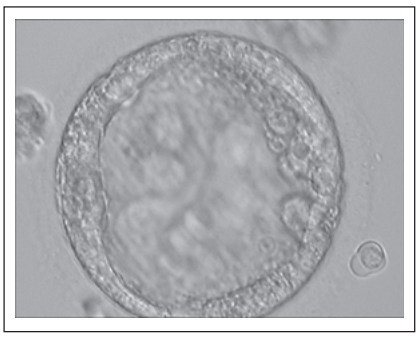

Figure 6: Trophoblast vesicle without inner cell mass consisting of only a minor number of trophectodermal cells.

gaps, the TE is scored b. The worst case scenario (grade c) would be a trophectoderm consisting of very few larger cells with a low number of tight junctions (Fig. 4).

Kovacic et al. [27] summarized all blastocysts showing an impaired trophoblast (e.g. extremely flattened cells, no sickle-shaped cells, no junctions between TE cells, cells with granulation, pigmentation and/or vacuolization) and noted a 36% implantation rate. This is in the line of others [11]. These authors counted trophectodermal cells around an embryonic equator in one plane of focus and could not find any difference between the mean number of TE cells (per plane) and the occurrence of a clinical pregnancy.

Thus, it could be speculated that any preliminary reduction in trophectodermal cell number could easily be compensated by the accelerated growth rate from day 6 onwards [15].

This would be contrary to the data of others [28] who observed that a decrease in the quality of trophectoderm had an almost linear relationship to a reduced rate of live births. In more detail, these authors reported a 64% live birth rate if the trophectoderm was classified as a and only 13% in the presence of a c-trophectoderm.

As an illustration it may be emphasized that a hatching blastocyst of optimal quality showing both an ICM and a TE of optimal cohesiveness would be scored grade Vaa (Fig. 1).

Bad Quality Blastocysts

Though numerous studies have evaluated the outcome of transfer of good quality blastocysts only a limited number of papers analyzed the fate of bad quality blastocysts [27, 29]. This group of blastocysts with poor prognosis usually shows lower cell numbers and a higher degree of chromosomal aberrations [29]. Bad quality blastocysts consist of numerous different morphological subtypes, such as blastocysts with excessive fragmentation, excluded blastomeres, and necrotic cells (Fig. 5) as well as trophoblast vesicles (Fig. 6).

The latter type is characterized by the absence of the inner cell mass [30] and a rather rudimentary trophectoderm (with only a minor number of nuclei); thus, it is more or less a trophoblastic vesicle with a dominant blastocyst cavity that also could be a large vacuole [27]. Only 3 out of 26 trophoblast vesicles implanted (11.5%) after transfer; however, one abortion reduced live birth rate to 7.7% [27].

The fate of all the other inferior blastocyst variations, though they may show acceptable ICMs or TEs, will also be compromised by larger amounts of fragments or excluded blastomeres that, on the one hand, will be associated with reduced cell numbers on day 5 [22] and, on the other, may interfere with hatching process per se [31]. Live birth rates (approximately 17%) in these bad quality blastocysts were only marginally increased [27] as compared to trophoblast vesicles.

Slightly better results could be achieved when blastocysts showing necrotic foci in one of the cell lineages had to be transferred. Assessing the actual location of such degenerative areas it turned out that outcome was worse if ICM was affected (23% live births) and slightly better if only TE was affected (32.8%). Consequently, ICM compactness and multicellularity contributed more to vital implantation than TE cohesiveness [27].

Similar to necrotic areas vacuoles at blastocyst stage could have a detrimental effect on developmental capacity (Fig. 5). In a recent study, 15.2% (36/237) of day 5 blastocysts showed vacuoles [32]. Interestingly, a statistically significant trend ($p = 0.011$) towards an elimination of vacuoles from the inner cell mass could be observed [32] since the vast majority of vacuoles could be located in the trophectoderm [33] indicating that, theoretically, embryos can develop strategies to minimize a negative impact of vacuolization on implantation behaviour. Reports on pregnancies achieved after transfer of vacuolized blastocysts are scarce; however, prognosis was much better if vacuolization was restricted to the TE [32].

Another important characteristic in terms of implantation behaviour of blastocysts is the presence of cytoplasmic extensions bridging the blastocyst cavity (Fig. 7) at the expanded or later stages (grades IV–VI). These processes are commonly present in half of the junctional TE cells spanning the boundary between the polar and the mural region of TE and are directed towards the blastocoelic surface of the ICM [34]. The cytoplasmic extensions are thought to be related to the polarized flow of cells from the polar to the mural trophectoderm, consequently they tend to withdraw as the cells reach their final location. Interestingly, variations in both shape (from broad triangles to string-like projections) and length (some fail to reach the ICM surface) have been observed [34].

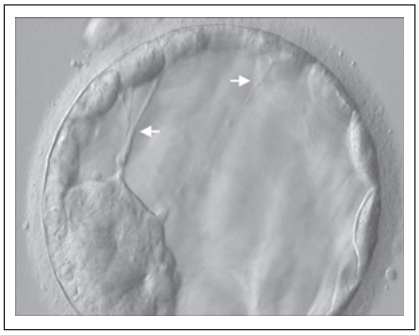

Figure 7: Expanded blastocyst (IVaa) showing characteristic cytoplasmic strings (arrows) within the blastocoel.

Figure 8: Expanded blastocyst (IVaa) that collapsed prior to spontaneous hatching ("breathing")

Table 2: Implantation and perinatal outcome of transfers with embryos hatching at different spots around the zona pellucida. Modified according to Ebner et al. [38].

	haching from embryonic pole	hatching from TE
n	32	84
positive β-hCG	22 (68.8%)	46 (56.8%)
clinical PR	22 (68.8%)*	39 (46.4%)*
birth	19 (59.4%)	34 (40.5%)
IR	27/50 (54.0%)*	52/140 (37.1%)*
babies born	20	37
gestation week	38.7 ± 2.5	38.2 ± 2.8
weight	2976 ± 693	2947 ± 669
sex ratio (m/f)	1.22	1.18
malformations	0	3 (8.1%)

* $p < 0.05$; IR: implantation rate; n: number of patients; PR: pregnancy rate; TE: trophectoderm

Whereas cytoplasmic extensions covering the surface of the inner cell mass are a common feature in early blastocysts, most of the surface usually is unaffected in expanding blastocysts [34]. Persistence of cytoplasmic strings up to expanded stage possibly marks blastocysts of developmental lability. It could indeed be an indicator of polarization breakdown, e.g. caused by poor media conditions, resulting in reduced embryo quality and impaired implantation rates [35].

Spontaneous Hatching

Regardless of its respective quality every blastocyst surviving until transfer day is forced to escape from its zona pellucida (ZP) in order not to face total arrest of development.

While an uterine influence on hatching behaviour is likely to exist in vivo [36], in vitro spontaneous hatching of the human embryo is rather supported by the tremendous increase of internal pressure caused by both a gradual accumulation of blastocoelic fluid and cellular proliferation, mostly of trophectodermal origin.

In the absence of the uterine milieu the hatching process in vitro starts with small vesicles protruding through the zona pellucida. It should be kept in mind that this blebbing does not necessarily indicate the precise location of subsequent hatching [33]. However, once a small opening has been created the TE starts to herniate and – governed by trophectodermal projections – a larger opening is created by mechanical forces. Electron microscopic findings show that specialized plump cells, called zona-breaker cells [31], line both sides of the trophectoderm at theoretical hatching spots. Superficial microvilli and bundles of contractile tonofilaments enable these specialists to interact with the ZP, somewhat acting like a sphincter. Additional mechanical help may come from the phenomenon of blastocyst "breathing" [33], a sequence of rapid collapses and slow re-expansions considered to assist final extrusion from the ruptured ZP (Fig. 8).

Though the main driving force during hatching is a mechanical one, cellular ultrastructure of the zona-breaker cells, especially the presence of secretory vesicles such as lysosomes, strongly indicates that biochemical processes are involved as well. This is in line with previous work stating that the hatching process may also be mediated by zona lysins [37].

In humans, hatching occurs at various regions of the zona pellucida. While some authors postulate that blastocysts show hatching sites mainly close to the ICM [33], others present contradicting data, finding that most of the blastocysts hatch from the abembryonic pole [31]. Considering the proportions within a blastocyst, the likelihood of blastocysts to hatch from the smaller embryonic site is much lower than the chance to herniate near the rather extensive mural trophectoderm. As a matter of fact, a recent study [38] supports the latter theory since out of all hatching blastocysts (Grade V) only 38.9% showed a zona breach close to the embryonic pole.

Interestingly, Table 2 shows a significantly higher implantation rate if blastocysts were transferred that hatched close to the embryoblast (54%) as compared to blastocysts hatching from the abembryonic pole (37%). Though, obstetric and neonatal outcome of these single blastocyst transfers did not differ, it seems that human blastocysts have a developmental benefit if they hatch adjacent to the ICM, since this area corresponds to the cells (syncytiotrophoblast) that will later drive invasion into the endometrium. Theoretically, hatching close to the ICM would 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 impaired or delayed if herniation takes place opposite the ICM and/or if hatching difficulties occur.

Morphology after Vitrification

Since more detailed blastocyst scoring systems allow for better prediction of implantation behaviour, steady reduction of the number of transferred blastocysts is recommended [9]. At the same

time, this strategy increases the number of supernumary blastocysts in culture that have to be stored in liquid nitrogen if the quality of these day 5 concepti is promising in terms of subsequent cryosurvival.

Some 15 years after the successful cryopreservation of a human blastocyst [39] two major approaches are currently applicable in routine IVF work. Slow freezing is a safe and feasible option in human blastocyst cryopreservation and results in adequate survival and pregnancy rates [40, 41]. However, since vitrification offers some obvious benefits compared to slow freezing, reports favoring this rapid freezing technique have become more frequent in literature [42–44] indicating that for blastocysts it is equivalent [45] or even better [46, 47] than slow freezing.

Several factors (unrelated to vitrification method) are known to directly influence the fate of a cryopreserved blastocyst after thawing/warming and transfer. It is important to realize that survival rates in literature are hardly comparable due to the fact that some embryologists focus on immediate survival while others suggest an additional waiting period of 24 hours to facilitate control of growth [43]. Differences in implantation rates may be attributed to the fact that not all working groups apply assisted hatching to the thawed blastocysts (whilst shrunk), though this was found to improve outcome [43].

Most importantly, morphology of the blastocyst will have a significant impact on survival [43]; therefore, only blastocysts with good to moderate cell lineages will usually be considered for cryostorage. Nevertheless, cryosurvival of morphologically inconspicuous blastocysts may also fail if they derive from a cohort of bad day 3 quality embryos [43].

A recent publication [48] introduced a grading system based on re-expansion, hatching (out of the artificial gap in the zona pellucida), cytoplasmic granulation and presence of necrotic foci.

Re-expansion and Hatching

It became evident that the efficiency of the vitrification method depends on the expansion of the blastocyst, with better survival in morula or early blastocyst stages compared to full or expanded blastocysts [48–51]. This phenomenon is possibly related to the size of the blastocyst cavity which in turn is correlated to the volume of watery liquid within the blastocyst.

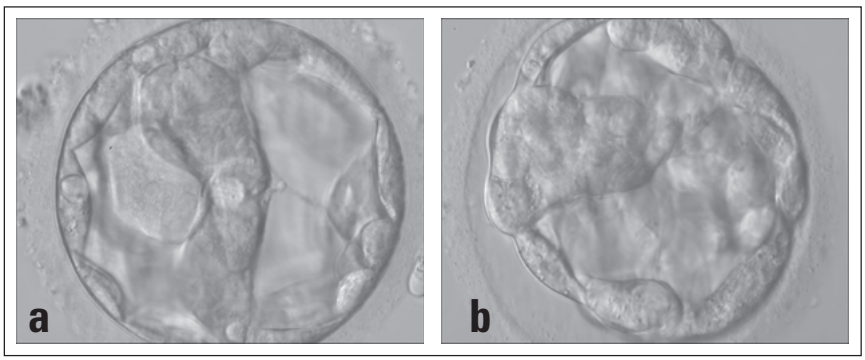

Figure 9: Expanded blastocyst (IVaa) before vitrification **(a)**. Full re-expansion is not reached 2h after warming **(b)**.

Due to the presence and size of the blastocyst cavity, vitrified-warmed blastocysts experience several morphologic changes and become collapsed during cryopreservation process. Thus, it is more difficult to score a vitrified blastocyst after warming than a fresh one [52].

Re-expansion of the blastocyst cavity (and consequently the blastocyst) after thawing/warming is expected within 24 hours [41, 43]. In slow-freezing approximately half of the frozen/thawed blastocysts (197/402) turned out to re-expand immediately after 2–4 hours in culture [52]. In addition, in vitrified/warmed blastocysts, failure of re-expansion process was found to be associated with significantly reduced rates of implantation ($p = 0.022$) and clinical pregnancy ($p = 0.021$) suggesting fast blastocoelic re-expansion of the cavity as a discriminative marker of viability [48].

Even if it can be assumed that after several more hours all thawed blastocysts will re-expand (Fig. 9) any delay in this process could be the manifestation of altered osmotic and/or metabolic conditions. These events are comparable to the situation found during blastocoele development for the blastocyst cavity when water enters the embryo cavity via tight junctions either diffusing passively or being pumped actively [33]. It can be hypothesized that in blastocysts with delayed re-expansion vitrification may have influenced its permeability to water. This could either be due to a cryoartefact per se or to laser manipulation during assisted hatching, though the latter assumption is less probable since zona drilling usually is performed immediately after warming when the blastocyst is shrunk. With respect to this, it is noteworthy that the rate of complete hatching is significantly higher if assisted hatching is performed near the inner cell mass as compared to the opposite region [53].

One morphological feature of thawed/warmed blastocysts that often goes with re-expansion is precocious hatching of the blastocyst through the artificial gap created by laser pulses. Quite logically, the probability of blastocysts to hatch is likely to be increased if the conceptus is already re-expanded. However, even shrunk blastocysts tend to leave their outer shell if the position within the zona is close to the opening [38]. Data from literature [38] suggest that double clinical pregnancy rates can be achieved (63%) if transferred blastocysts already started to hatch compared to blastocysts without (29%) this morphological attribute. When both positive prognostic markers, re-expansion and hatching, were combined, every second blastocysts (52.2%) implanted. Blebbing out of the artificial gap (2 hours after warming) obviously characterizes a subgroup of blastocysts that can hatch completely without being trapped within the zona [53].

Necrotic Foci and Cytoplasmic Defects

In contrast to previous positive predictors, necrotic foci in warmed blastocysts are considered to have a negative impact on further development. Like in freezing at earlier cleavage stages areas of necrosis in thawed blastocysts mark cells that did not survive vitrification.

There is general agreement that at earlier stages at least 50% of the blastomeres have to survive in order to call a thawed embryo viable [21, 54]. Recently, it could be shown that blastomere loss after thawing in day 2 embryos has a detrimental effect on blastocyst formation and cell number [21]. Partially damaged day 3 embryos can be rescued if the necrotic blastomeres are removed prior to transfer [54, 55]. However, prior to compaction cell-cell adhesion can be neglected and does not reflect cell fusion in blastocysts at all. In other words, removal of necrotic cells is rather impossible on day 5 and would possibly harm the affected cell lineages.

Ebner et al. [48] provided first evidence that partial damage of the blastocyst caused by vitrification does not reduce rates of implantation and pregnancy. This is all the more interesting since it played no role whether ICM was affected or TE. In detail, of those blastocysts with known outcome 44% (7/16) implanted if the trophectoderm was marginally degenerated compared to 37% (10/27) if minor parts of the ICM showed necrosis ($p > 0.05$). Obviously, blastocysts can compensate for minor injuries.

This is not the case if the whole cytoplasm is harmed by vitrification. Occasionally, blastocyst show extensive granular cytoplasm immediately after thawing instead of an expected homogeneous appearance. Though these blastocysts could be considered for transfer once they have recovered, their implantation potential seems to be limited.

This morphological conspicuousness is different from previous cytoplasmic irregularities such as cytoplasmic pitting, manifestation of which is probably culture dependent [56]. The granular cytoplasm sometimes observed after vitrification is characterized by a halo-like structure in the periphery of the cells (which is most likely to consist of water) and a dominant accumulation of some cytoskeletal components. Warmed blastocysts showing this phenomenon have a reduced capacity to survive vitrification. In the presence of this cytoplasmic anomaly, blastocysts with good prognosis for subsequent survival may be distinguished from those with bad prognosis by healthy appearance of the cell membranes [57] which should be a small intact line.

Conclusion

Blastocyst culture as the method of choice for all patients is a balancing act and could result in relative high rates of cycle cancellation. One prerequisite for optimizing blastocyst culture, transfer, and cryopreservation is to develop a better understanding of morphological criteria that may influence the implantation potential of a certain blastocyst. Numerous patient- and oocyte/embryo morphology related criteria have been suggested that allow for identification of a certain subgroup of patients who would definitely benefit from prolonged culture.

Usually, several blastocysts of different qualities can be grown per patient which makes an optimized blastocyst scoring system indispensable. With the obvious current trend towards a significant reduction of transferred embryos, every effort should be made to filter out the blastocyst with the highest implantation potential. With respect to this, it is recommended to weight the morphological criteria at blastocyst stage as follows: quality of the inner cell mass, quality of the trophectoderm, expansion (e.g. blebbing out of the zona at the embryonic pole), few anomalies (excluded blastomeres, vacuoles, necrotic foci, cytoplasmic strings).

It should be obligatory to cryopreserve supernumary blastocysts of good to moderate quality in order to increase cumulative pregnancy rate. With regard to this it is very important to achieve a reproducible outcome, especially in terms of survival after thawing, to allow high success rates after frozen/vitrified blastocyst transfer.

According to Van den Abbeel et al. [41] the evaluation of immediate blastocyst survival on the basis of morphology is difficult and highly subjective, however, a new scoring system (re-expansion, hatching through artificial gap, cytoplasmic appearance) proved useful in order to predict both rates of pregnancy and implantation [48].

Conflict of Interests

The authors certify, that there is no conflict of interests in relation to this article.

Relevancy to Practice

To conclude, combining both fresh single blastocyst transfer and single or double frozen blastocyst transfer would not only help to keep the overall hormonal dosage applied to a patient to a minimum but also to reduce the number of blastocysts per transfer which would result in a significant reduction of multiple pregnancy rate and a higher cumulative pregnancy rate. This would assist to further increase acceptance of this method in both patients and clinicians/embryologists.

Relevanz für die Praxis

Ein exaktes Scoring der Blastozyste vor und nach der Kryokonservierung erlaubt eine gute Prognose hinsichtlich einer Implantation. Dadurch kann die Zahl der zu transferierenden Embryonen weiter reduziert werden, was einem Hauptproblem der IVF, den Mehrlingsschwangerschaften, entgegenwirkt.

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