Comparison of human blastulation rates and total cell number in sequential culture media with and without co-culture

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Recent interest in delayed embryo transfers necessitated the evaluation of two improved in-vitro systems that could generate viable blastocysts. A total of 178 two-pronucleated embryos (entire cohorts) from 19 patients was cultured in IVF50 medium (100 µl) under oil for 24 h until day 2. Each patient’s day 2 embryos were then equally allotted to two in-vitro systems. Embryos in system A were grown until the morning of day 3 on Vero cells covered with IVF50 medium (100 µl) under oil. The medium was then replaced on day 3 with a 1:1 mixture (100 µl) of IVF50:S2 medium and on day 4 with S2 medium only. The same culture protocol was used for system B without Vero cells. Throughout the 5 days all dishes were housed in sealed humidified modular chambers containing a triple gas atmosphere. Separately, 175 spare embryos from 80 patients were grown in system A and B up to days 6 and 7 for total cell number (TCN) analysis. Blastulation rates were not significantly different between system A and B (67.4 versus 68.5%; \( P > 0.01 \)) although co-cultured embryos cleaved slightly faster by day 4. The overall pregnancy and implantation rates were 52.0% and 32.1% for the 19 patients each of whom received a mixed cohort of three day 5 embryos from both systems. TCN values for the day 6 and 7 blastocysts from both systems were high and increased steadily from days 6–7 and from expanded to hatching stages. There were no significant differences in TCN for day 6 expanded blastocysts between the two systems although day 6 hatching and hatched co-cultured blastocysts had greater values than non-co-cultured blastocysts (246.0 ± 18.5 and 236.7 ± 17.8 versus 173.0 ± 13.5 and 166.5 ± 16.0; \( P < 0.01 \)). The results demonstrated that the culture protocol using the sequential IVF50–S2 media combination was a good substitute for Vero cell co-culture for the transfer of viable day 3–6 embryos.

**Key words:** blastocyst/sequential culture media/total cell number/Vero cells

**Introduction**

There has been tremendous interest in improving the results of assisted reproduction and understanding implantation through zona-intact and zona-free blastocyst transfers (Scholtes and Zeilmaker, 1996; Fong et al., 1997, 1998; Gardner et al., 1998; Jones et al., 1998). From these recent studies it appears that implantation rates through blastocyst transfer are quite high and suggestions have been made to replace at this stage only one to two embryos so as to avoid the incidence of triplets and reduce twinning. In fact, Scholtes and Zeilmaker (1996) claimed that an implantation rate of 25% could be obtained after transfer of one to two zona-intact blastocysts. Blastocyst transfer assumes that reasonable in-vitro systems have been developed that can consistently generate high blastulation rates. These new systems have involved changes to gas environment and protein composition for embryonic growth, the use of helper cell monolayers (co-culture) and the development of new generation sequential culture media combinations.

Early studies showed that the omission of glucose at early cleavage stages and the maintenance of pyruvate in simple culture media result in blastulation rates of nearly 59% (Hardy et al., 1989; Conaghan et al., 1993). Embryo development to the 8-cell stage was enhanced when pyruvate was maintained and glucose omitted from a simple medium while the resulting blastocysts had significantly higher total cell numbers (TCN) compared with embryos grown in the presence of glucose (99.1 versus 58.4) (Conaghan et al., 1993). It has been shown that there is a switch in substrate preference at the 8-cell/ morula stage in favour of glucose, which becomes the predominant energy source at the blastocyst stage (Gardner and Leese, 1986). Pyruvate uptake increases significantly over the third and fourth cleavage divisions and peaks at the morula stage. Between the morula and blastocyst stages there is a significant decline in pyruvate uptake while between the 16-cell stage and blastocyst there is a significant increase in glucose uptake. Several important events occur during the switch from pyruvate-based to glucose-based metabolism. The embryonic genome is activated between the 4-cell and 8-cell stages (Braude et al., 1988). At this stage, accompanying the events of transcription are changes in the types of proteins that are synthesized. Glucose is a precursor of many macromolecular cell constituents such as the ribosome moieties for nucleic acid synthesis, glycerol phosphate for the formation of phospholipids and complex sugars in mucoproteins and mucopolysaccharides and it has been suggested that the switching on of the embryonic genome may impose an increased requirement for glucose (Leese, 1990). Furthermore when the inner cell mass (ICM) and trophoblast (TE) of the blastocyst undergoes cell differentiation involving a change in cell function and morphology there would be concomitant changes in metabolism, and the appearance of the first differentiated cell type coincides with a decline in pyruvate uptake and sharp increase in glucose uptake (Hardy, 1993). Thus, if embryos were to be grown to the blastocyst stage, a single medium...
(simple or complex) may not be able to fulfil all these complex metabolic requirements for 5 days.

As such, several co-culture systems were developed that repeatedly showed high blastulation rates ranging from 55–69% (Bongso et al., 1989; Menezo et al., 1992; Wiemer et al., 1993). Helper cell monolayers were developed because the simple and complex culture media available at that time were not optimal enough consistently to yield blastocysts for transfer. However, no statistically significant improvement in early human embryogenesis in the Vero cell co-culture system was reported by Van Bl erkom (1993), and Sakkas et al. (1994) demonstrated high blastocyst rates but no differences in pregnancy rates in a Vero co-culture system when compared with T6 medium supplemented with 10% maternal serum.

Recently, alternative approaches to improving in-vitro systems have been developed where embryos are either grown in a mixture of simple and complex culture media throughout the 5 days (e.g., Earle’s and Ham’s F12 media; Scholtes and Zeilmaker, 1996) or embryos are grown sequentially for the first 2 days in a simple medium and then transferred to a complex medium from day 3 onwards with or without co-culture. Sequential culture media combinations were proposed because, as described earlier, the metabolic requirements of embryos change from one stage to another throughout the 5 days in vitro. In one study, embryos were grown to day 2 in IVF50 medium (Scandinavian IVF Science AB, Goteborg, Sweden) and from day 3–5 in G2 medium (Jones et al., 1998) while in another study by our group, embryos were grown in IVF50 medium (for the first 2 days), then transferred to a mixture of 50% of IVF 50 medium and 50% of S2 medium (Scandinavian IVF Science AB) on day 3 to prevent ionic shock to embryos, and then subsequently grown in S2 medium from day 4 onwards. Vero cell co-culture was used together with the sequential culture media without serum throughout the 5 days in the latter study (Fong et al., 1998). In another recent study, embryos were grown for the first 2 days in a complex culture medium [Ham’s F10 + 15% fetal calf serum (FCS) or G1 medium; Gardner, 1994] and then transferred to a super-complex medium (G2) from day 3 onwards (Gardner et al., 1998). In all these three recent in-vitro systems, blastocyst transfer resulted in improved implantation rates of 23% (Jones et al., 1998), 45.5% (Gardner et al., 1998) and 33.3% (Fong et al., 1998).

Now that improved sequential culture media formulations have been developed, it is urgent necessary critically to evaluate whether such cell-free systems will be as good or superior to co-culture systems before helper monolayers can be done away with altogether. This study compares the in-vitro behaviour of cavitated and non-cavitated embryos and the viability of blastocysts cultured in cell and cell-free systems, i.e. IVF50 and S2 culture media with and without Vero cell co-culture.

Materials and methods

Source of embryos

Nineteen patients with idiopathic or male factor subfertility admitted for in-vitro fertilization (IVF) were included in this study. Ethical approval was granted for this study by the Hospital Ethics Committee. The patients were superovulated with a down-regulation protocol of a gonadotrophin releasing hormone (GnRH) agonist (Suprefact, Allemagne, Germany) followed by follicle stimulating hormone (FSH) (Metrodin® HP; Serono, Geneva, Switzerland) or Gonal F® (Serono). Follicular growth was monitored by ultrasonography and measurement of oestradiol concentrations. When two dominant follicles reached 16 mm diameter, a dose of 10 000 IU of human choric gonadotrophin (HCG) (Profasi®; Serono) was administered to trigger ovulation. At 36 h after HCG, oocytes were recovered transvaginally using ultrasound guidance and ASP250 flushing medium (Scandinavian IVF Science AB). ASP250 is composed of human tubal fluid (HTF) as a basal medium supplemented with heparin and HEPES buffer. All mature oocyte cumulus complexes (OCC) were washed in IVF50 medium (Scandinavian IVF Science AB) and each OCC pre-incubated for 3–6 h in 0.5 ml of IVF50 medium without oil in 5 ml loosely capped tubes at 37°C in a 5% CO2 in air atmosphere.

Motile spermatozoa separated by a 40:70:90 discontinuous colloidal silica gel triple gradient (Puresperm, Nideacon, Gotteborg, Sweden) were used to inseminate oocytes or for intracytoplasmic sperm injection (ICSI) (Ng et al., 1993). For insemination, each OCC was introduced into 100 µl sperm droplets in IVF50 medium containing 10 000 motile spermatozoa under oil (Ovoil®; Scandinavian IVF Science AB) in 4-well plastic dishes (Nunclon, Roskilde, Denmark) and incubated at 37°C in a humidified modular chamber (Billups-Rothenberg, Delmar, CA, USA) with a controlled environment of a 5% CO2, 5% O2 and 90% N2 gas atmosphere. The desiccators were housed in large 5% CO2 incubators (Heraeus, Hanau, Germany). At 16–20 h after insemination, oocytes were mechanically denuded of cumulus cells and fertilization determined by the presence of two pronuclei and two polar bodies.

Growth of embryos in two in-vitro systems

Two-pronucleate embryos of all patients were cultured in IVF50 medium (100 µl) under oil for 24 h until the 4-cell stage (day 2). The entire cohort of each patient’s 4-cell stage embryos were equally allotted to two in-vitro systems for further embryonic growth on the morning of day 2. Embryos in system A were grown until the morning of day 3 on Vero cells (plated on the day of oocyte recovery with 5 × 10⁴ cells per well in 4-well plastic dishes), covered with IVF50 medium without serum and under oil. On the morning of day 3, IVF50 medium was replaced with a mixture of 50% IVF50 and 50% S2 medium (without serum) and on days 4 and 5 the embryos were grown in S2 medium alone. The same monolayer was used throughout the 6 days of culture. Monolayers were only 50% confluent on the day of initial plating (day of oocyte recovery) so as to provide space for the growth of cells throughout the 6 day period. The cells were actively mitotic during this entire period providing for ample secretion of potential growth factors by the cells and negative conditioning of the medium. The same culture protocol was used for system B except that cells were not used and embryos were grown in the sequential culture media alone with daily manipulations carried out at the same time as system A. All 4-well dishes in both systems were housed in sealed humidified modular chambers containing the triple gas mixture throughout the 5 days.

Monitoring of embryos

Embryos in both systems were monitored daily at the same time in the morning using Nomarski and bright field inverted optics for cleavage speed, fragmentation, regularity of blastomeres, compaction, cavitation and expansion for 5 days before transfer or cryopreservation. The incidence of 6- to 10-cell, compacting, compacted, early and late cavitating and blastocyst (early, expanding,
Figure 1. Stages of embryonic development observed in both in-vitro systems; (A) day 4 compacting embryo on Vero cells + S2 medium; (B) day 4 compacted embryo in S2 medium only; (C) day 4 early cavitating embryo in S2 medium only (blastocoele just appearing); (D) day 5 late cavitating embryo on Vero cells + S2 medium [blastocoele larger but no distinct inner cell mass (ICM) and trophoderm (TE) laid down as yet]; (E) day 5 early blastocyst on Vero cells + S2 medium (ICM and TE laid down and embryo diameter usually similar or slightly larger than late cavitating embryos); (F) day 5 expanding blastocyst in S2 medium only (diameter not fully expanded as yet). Scale bar = 0.03 mm (all panels).

expanded) stages were recorded. The appearance and description of the various stages of embryonic development are given in Figure 1 and Table I respectively. Good blastocysts (early, expanding and expanded) were classified as those with a clear distinct ICM, a well laid down TE with sickle-shaped cells, a single large blastocoelic cavity and a thin zona pellucida. Early, expanding and expanded blastocysts were distinguished on diameter size. Early blastocysts had the same diameter as early and late cavitating embryos while expanding and expanded blastocysts had gradual increases in diameter (Figures 1, 2 and 3). Bad blastocysts were those that did not conform to these markers (Figure 3A, B). Using these markers up to three good blastocysts per patient were selected for transfer, some were frozen at the patient’s request and the remaining donated for TCN studies. All 19 patients in this study had day 5 transfers with each patient receiving a mixture of three co-cultured and non-co-cultured embryos. It was therefore not possible to obtain the pregnancy rates for embryos in each group.

**Total cell number**

In a separate experiment 80 patients donated 175 of their spare embryos (110 good and 65 poor embryos) for studies on TCN. Good and bad quality was defined using the same morphological markers described in the first experiment. These embryos were grown in the same two culture protocols (system A and B) as in the previous experiment except that they were monitored longer until day 7. Photographs were taken of good (expanded, hatching and hatched) and bad blastocysts on days 5, 6 and 7 and the same blastocysts were analysed for TCN from the two in-vitro systems so as to evaluate their viability comparatively (Figure 2).

All blastocysts in the two systems were processed for TCN at the same time as the embryos in the first experiment were recorded for cleavage speed and morphological characteristics. For obtaining TCN, each blastocyst was first photographed and then placed in 0.5% sodium citrate and incubated at 37°C for 15–30 min. The embryo was then placed on a precleaned grease-free glass slide using a fire polished Pasteur pipette and most of the sodium citrate solution was then aspirated. A few drops of fixative (1:3, glacial acetic acid: methanol) delivered from a 1 ml tuberculin syringe with a 25 gauge needle was placed directly over the blastocyst, allowing it to swell and flatten. Excess fixative was avoided to prevent overscattering of nuclei. The slides were air dried and stained with Giemsa in phosphate buffer. Each embryo was photographed and the total number of cell
The results of this study demonstrate that since blastulation rates and TCN were not significantly different between the two in-vitro systems and the fact that pregnancy and implantation rates were very high after transfer of blastocysts from both in-vitro systems (52.0% and 32.1% respectively), the culture protocol using sequential IVF50 and S2 media without cells was as good as co-culture and therefore the media combination could replace Vero cell co-culture for blastocyst transfer in the human. It is important to note that the blastulation rates receiving mixed embryos in this study and the final implantation rates were 32.1%.

The TCN for good and bad embryos are shown in Figures 2 and 3 and Table III. Although the TCN for the good expanded blastocysts on day 6 were similar in both systems ($P > 0.01$), day 6 (a.m.) hatching and hatched co-cultured blastocysts had significantly greater mean TCN of 246.0 ± 18.5 and 236.7 ± 17.8 when compared to values for good blastocysts grown without co-culture (173.0 ± 13.5 and 166.5 ± 16.0; $P < 0.01$). The TCN for day 7 (a.m.) expanded, hatching and hatched blastocysts were not significantly different between the two in-vitro systems (233.3 ± 19.0 to 311.4 ± 21.1 versus 227.1 ± 17.1 to 286.0 ± 13.5, $P > 0.01$). TCN values increased steadily from day 6 to day 7 and from expanded to hatching stages while values decreased slightly from hatching to hatched stages. The TCN for good blastocysts of all three stages (expanded, hatching and hatched) were significantly greater than the values for poor blastocysts in both in-vitro systems (160.9 ± 15.1 to 311.4 ± 21.1 versus 43.7 ± 4.1 to 131.5 ± 14.1 and 166.5 ± 16.0 to 286.0 ± 13.5 versus 64.3 ± 6.0 to 88.9 ± 7.2; $P < 0.001$). One day 7 co-cultured hatching blastocyst had a TCN of 543 and one day 6 expanded non-co-cultured blastocyst had a TCN of 612. These two blastocysts were not included in the calculations of means in Table III as they were extraordinarily high.

### Discussion

The results of this study demonstrate that since blastulation rates and TCN were not significantly different between the two in-vitro systems and the fact that pregnancy and implantation rates were very high after transfer of blastocysts from both systems (52.0% and 32.1% respectively), the culture protocol using sequential IVF50 and S2 media without cells was as good as co-culture and therefore the media combination could replace Vero cell co-culture for blastocyst transfer in the human. It is important to note that the blastulation rates
Figure 2. Good day 7 blastocysts (panels A, C, E) with corresponding total cell numbers (TCN) observed in both in-vitro systems. For TCN analysis, each embryo was exposed to 0.5% sodium citrate at 37°C for 15–30 min followed by fixation on a glass slide using 1:3 glacial acetic acid: methanol and then stained with Giemsa. (A, B) day 7 expanded blastocyst on Vero cells + S2 medium with a TCN of 214; (C, D) day 7 hatching blastocyst in S2 medium with a TCN of 272; (E, F) day 7 hatched blastocyst on Vero cells + S2 medium with a TCN of 307. Scale bars: A, C = 0.03 mm; B, D, F = 0.1 mm; E = 0.05 mm.

observed in this study (67.4–68.5%) were based on the entire cohorts of patients’ fertilized oocytes and not on spare embryos as in other studies (Bolton et al., 1989; Desai et al., 1997). This therefore reflects a realistic measure of the two in-vitro systems. TCN has been considered to be the most sensitive assessment of development capacity (Brinsko et al., 1994) and since the TCN of blastocysts generated from the sequential culture media alone were as high as those from co-cultured blastocysts (Table III), the non-co-cultured blastocysts are expected to implant equally well as the co-cultured blastocysts.

The use of this sequential culture medium protocol saves the labour and time involved in the plating of the Vero cells for co-culture and also avoids any possible risk of viral cross contamination. Interestingly, some of the embryotrophic factors released by co-culture cells (taurine, glutamine, insulin) have been introduced into S2 medium making its beneficial effects comparable to co-culture. It is important to note however that this study compared a commercial non-physiological established cell-line (Vero cells) for co-culture which is easily available, purified and known to induce positive co-culture effects (Ménézo et al., 1992). Physiologically, to mimic the in-vivo tubal environment it would have been better comparatively to evaluate human tubal cells as the co-culture system with the sequential culture media combination (IVF50-S2) of the present study. Recently, several embryotrophic factors such as colony stimulating factor-I, leukaemia inhibitory factor, epidermal growth factor and interleukin-2 were shown to be released by human tubal cells in vitro (Barmat et al., 1997). Thus, human tubal cells may have generated more beneficial effects when compared to Vero cells. The best co-culture effects are observed when human tubal cells are used between the first and fourth passage. However, human serum is necessary to keep such early passaged tubal cells alive and plated through 5 days unlike Vero cells which are from an established cell line at the 120th passage (Bongso et al., 1995). The growth of tubal cells without serum for 5 days results in
Human blastulation in culture

Figure 3. Poor day 7 blastocyst (A) in S2 medium with a total cell number (B) of 89; (C) day 5 embryos (late cavitating embryo, early and expanding blastocysts) observed on Vero cells + S2 medium. Note gradual increases in diameter; (D) day 5 embryos on Vero cells + S2 medium with different cleavage speeds. Note two embryos arrested at the compacted stage and four embryos cleaving regularly at the late cavitating and blastocyst stages. Scale bars: A = 0.03 mm; B, C, D = 0.1 mm.

Table II. Human embryonic behaviour in two in-vitro systems

<table>
<thead>
<tr>
<th>In-vitro system</th>
<th>Day 2 2–4 cells</th>
<th>Day 2 6–10 cells</th>
<th>Day 3 Compactingb</th>
<th>Day 4 Compacting Compacted Early cavitating</th>
<th>Day 5 Cavitated embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-culturec and sequential media</td>
<td>89</td>
<td>59/89 (66.3)</td>
<td>17/89 (19.1)</td>
<td>23/89 (25.8)</td>
<td>38/89 (42.7)</td>
</tr>
<tr>
<td>Sequential media alone</td>
<td>89</td>
<td>62/89 (69.7)</td>
<td>9/89 (10.1)</td>
<td>19/89 (21.3)</td>
<td>41/89 (46.1)</td>
</tr>
</tbody>
</table>

| Day 5 Late cavitating Early blastocyst Expanding blastocyst Total |
|------------------------|-----------------|-----------------|-------------------|-------------------------------|
| 37/89 (41.6)           | 13/89 (14.6)    | 12/89 (13.1)    | 61/89 (68.5)f      |

a19 patients.
bEmbryonic stages described in Figure 1.
cVero cells + IVF50–S2.

Figures in parentheses are percentages: $^{(a)} p < 0.05$, $^{(b)} p > 0.01$.

Cell senescence and detachment of cells from the plastic causing toxic effects to the embryos (Bongso, 1996). It was therefore not possible to compare human tubal cells with IVF50-S2 in this study as the use of serum with its many ingredients to keep the tubal cells alive would have confounded the results of this study because IVF50 and S2 media contain only human serum albumin (HSA) as their protein source. Moreover, serum in culture media has been shown to have negative effects on blastocyst development (Gardner, 1994).

The ionic composition of IVF50 and S2 media is drastically different and as such it was important gradually to introduce the S2 medium to the embryos on day 3 by using a mixture of 50% IVF50 and 50% S2 as recommended by the manufacturers (P. Svalander and P. Holmes, Scandinavian IVF Science AB, personal communication). IVF50 and S2 are modified HTF media supplemented with HSA as a protein source. IVF50 is a simple medium containing salts, glucose, lactate, pyruvate and EDTA while S2 is a complex medium supplemented further with vitamins, hormones, amino acids and insulin. IVF50 has lower glucose and higher pyruvate concentrations compared to S2 (high glucose, low pyruvate) based on the shift from pyruvate to glucose-based metabolism when embryos are grown from early cleavage to blastocyst stages. Since the ionic composition is different between IVF50 and S2 and there is a need to use both media in a 1:1 ratio on day 3, the manufacturers have recently introduced S1 medium which has a similar ionic composition to S2. With the S1–S2 combination embryos are grown up to day 3 in S1 medium.
and then transferred to S2 medium on day 4 and 5 without the need of a 1:1 ratio on day 3 (P. Svalander and P. Holmes, personal communication). Surprisingly, the change from IVF50 directly to G2 (which are also significantly different in ionic composition) on day 3 in the study by Jones et al. (1998) did not appear to compromise blastocyst development. Perhaps these authors could have improved further their enviable implantation rates had they used a 1:1 mixture of IVF50:G2 on day 3 of embryonic development. Ionic shock may not have been expected in the study by Gardner et al. (1998) where embryos were transferred from one complex medium to another with almost the same ionic composition (G1 to G2).

It is interesting to note that both in-vitro systems in this study produced embryos with much higher TCN numbers on days 6 and 7 except for one embryo on day 7.

Using the culture protocol described in this study, implantation rates for day 3, 4 and 5 transfers were 26.0% (41 patients), 22.8% (21 patients), 33.3% (19 patients) when a mean of 2.4 ± 0.7, 2.8 ± 0.4 and 2.5 ± 0.6 embryos were replaced respectively (Bongso et al., 1999). Scott and Smith (1998) showed equally good implantation rates of (28%, 48 patients) but after the transfer of a mean of 3.7 ± 1.3 early 2-cell embryos with a corrected embryo score (total score/number embryos, CS) of ≥15. Scoring was based on the proximity of pronuclei, alignment of nucleoli and the presence of a cytoplasmic halo when embryos were at the two pronuclear stage. These selection criteria were based on a model of polarized parameters first suggested by Edwards and Beard (1997) and later confirmed by Antczak and Van Blerkom (1997). Scott and Smith (1998) endorsed that the only negative aspect of pronuclear or early 2-cell stage transfer was the fact that more embryos are transferred. Studies are in progress by our group to evaluate blastulation rates after pronuclear scoring using the polarized parameter model and the embryo culture protocol in the present study. Future comparative prospective randomized studies of implantation rates with early and delayed transfers of only two embryos using the polarized parameter model and other markers will provide us with more information.

### Table III. Total cell number (mean ± SD) of human blastocysts in two in-vitro systems

<table>
<thead>
<tr>
<th></th>
<th>Co-culture</th>
<th>Medium only</th>
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<tr>
<td></td>
<td>Good</td>
<td>Poor</td>
</tr>
<tr>
<td>Day 6 (a.m.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expanded</td>
<td>160.9 ± 15.1 (14)(^a)</td>
<td>43.7 ± 4.1 (6)</td>
</tr>
<tr>
<td>Hatching</td>
<td>246.0 ± 18.5 (10)(^c)</td>
<td>84.0 ± 6.2 (4)</td>
</tr>
<tr>
<td>Hatched</td>
<td>236.7 ± 17.8 (6)(^e)</td>
<td>–</td>
</tr>
<tr>
<td>Day 7 (a.m.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expanded</td>
<td>233.3 ± 19.0 (8)(^g)</td>
<td>85.3 ± 5.8 (12)</td>
</tr>
<tr>
<td>Hatching</td>
<td>311.4 ± 21.1 (12)(^i)</td>
<td>131.5 ± 14.1 (4)</td>
</tr>
<tr>
<td>Hatched</td>
<td>286.3 ± 15.4(8)(^k)</td>
<td>170.0 (1)</td>
</tr>
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</table>

\(^{a,b}P > 0.01; ^{c,d}P < 0.01; ^{e,f}P < 0.01; ^{g,h}P > 0.01; ^{i,j}P > 0.01; ^{k,l}P > 0.01.\)

Values in parentheses are number of embryos; poor embryos in both in-vitro systems did not hatch on days 6 and 7 except for one embryo on day 7.
on what the most ideal embryonic stage should be for transfer that will yield the most optimal implantation rates with reduced multiple pregnancies.

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Human blastulation in culture