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Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing

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BACKGROUND: The present study was undertaken to examine the usefulness of both vitrification and assisted hatching (AH) on blastocysts that originate from embryos showing different qualities during their cleavage stage. METHODS: A total of 281 blastocysts were vitrified (93 vitrification–warming cycles) in a mixture of ethylene glycol–dimethylsulphoxide–Ficoll and sucrose using the Hemi-Straw (HS) carrier system. After warming, AH using the partial dissection technique was performed in 36 cycles. RESULTS: After warming and culture for 24 h, a total of 168 blastocysts (60%) was suitable for embryo transfers and a total of 25 ongoing pregnancies (27%) was obtained. Forty-nine transfers of 96 no-AH blastocysts and 36 transfers of 72 AH blastocysts resulted in an implantation rate of 13 and 22% respectively ($P < 0.05$). The percentage of transfers with at least one hatching blastocyst was significantly higher after application of AH (69 versus 33%) ($P < 0.001$). In all, 73 and 38% of blastocysts showing respectively optimal and non-optimal embryo development during the early stage were available for transfer ($P < 0.001$). Consequently, implantation rates of 19 and 6% were obtained after transfers of blastocysts showing respectively optimal and poor embryo development. CONCLUSIONS: Artificial opening of the zona pellucida after warming of vitrified blastocysts significantly improved the rate of transfers with hatched blastocysts and the implantation and pregnancy rates. The percentage of blastocysts that survived the HS vitrification procedure and were available for embryo transfer is related to their previous developmental quality.

Key words: assisted hatching/cryopreservation/embryo quality/human blastocyst/vitrification

Introduction

Results with conventional slow-freezing methods for blastocysts have been variable, justifying investigation of alternative approaches. Recently, Kuleshova and Lopata (2002) reported that vitrification can be more favourable than slow cooling and has to be considered as an alternative to traditional slow rate.

Initially, vitrification was performed after loading cleavage stage embryos (Mukaida *et al*., 1998) or blastocysts in 0.25 ml insemination straws (Vanderzwalmen *et al*., 1997; 2002; Yokota *et al*., 2000; 2001). In this manner, the highest cooling rates were limited to ~2500°C/min.

Recently, particular attention has been paid to the cooling rate. The success rates of vitrification have been increased with the use of an ultra-rapid vitrification procedure. By highly increasing the cooling and thawing rates, the formation of ice crystals and chilling injuries are reduced. Ultra-rapid cooling procedures are based on direct contact between a reduced volume of cryoprotectant and liquid nitrogen (LN_2). Mammalian blastocysts are loaded on the surface of electron microscope grids (Martino *et al*., 1996), in an open pulled straw (Vatja *et al*., 1997), in a flexipet-denuding pipette (Liebermann *et al*., 2002), or in a cryoloop (Lane *et al*., 1999) before plunging them in LN_2.

Successful human pregnancies were reported after carrying out high speed vitrification methods of blastocysts using the cryoloop or the electron microscope grids (Choi *et al*., 2000; Mukaida *et al*., 2001; Cho *et al*., 2002; Reed *et al*., 2002; Son *et al*., 2003).

In order to accomplish vitrification of blastocysts, which requires an extremely fast cooling rate while considering the amount of space required for storage, we developed the HS (Vanderzwalmen *et al*., 2000). Recently, Liebermann and Tucker (2002) reported that the HS was easy to use and successful for vitrification of different stages of development (oocytes and day 3 embryos). The tip of the HS is designed to hold the blastocysts in a very small volume of vitrification solution allowing very fast cooling and warming rates as compared with those achieved by immersion of sealed 0.25 ml plastic straws into LN_2.

The initial clinical application of blastocyst vitrification using the HS resulted in a pregnancy outcome that was below our expectation despite acceptable rates of re-expansion 24 h

after warming. We noticed a difference in the pregnancy rates between transfers, which were performed with expanded blastocysts, or blastocysts that had hatched spontaneously. Twenty-two transfers with at least one expanded blastocyst resulted in only four pregnancies. On the other hand, 10 transfers with at least one spontaneously hatched blastocyst resulted in three pregnancies.

Considering these observations we wanted to know if the reduced ability of vitrified expanded blastocysts to further develop and implant lay in their inability to hatch out of zona pellucida (ZP).

This reduced capacity to hatch may be explained either by a hardening of the ZP or by leaving many embryos of questionable quality, and therefore of poor prognosis, for an extended period in culture.

Hardening of the ZP may occur spontaneously following fertilization, after prolonged exposure of human embryos to artificial culture conditions and also after cryopreservation (De Vos and Van Steirteghem, 2000). In the mouse, Carroll *et al*. (1990) observed that the freezing–thawing procedure induced changes in the ZP of oocytes which are responsible for a decrease in the rate of fertilization. To the best of our knowledge, only two reports analysed the effect of assisted hatching (AH) in conjunction with human frozen–thawed embryo transfers (Tucker *et al*., 1991; Check *et al*., 1996). Check *et al*. (1996) reported a clinical pregnancy rate of 13.7% after zona drilling on frozen day 3 embryos versus 5.3% in the control group. The clinical pregnancy rate after mechanical hatching of the ZP on day 2 embryos was 28 versus 15% in the control group, and the implantation rate was 16 and 9% respectively in the manipulated and intact ZP group (Tucker *et al*., 1991). Although, the number of cases did not allow these results to reach statistical significance, a tendency toward improved outcome after artificial opening of the ZP justifies further investigation in this field.

The second factor that may interfere with the hatching process concerns the quality of the blastocyst. Although some blastocysts showed a normal morphological aspect, they originated from a group of embryos with sub-optimal morphology on days 2 and 3. Probably these blastocysts have a lower potential to be successfully cryopreserved and/or to proceed to a spontaneous hatching.

According to Balaban *et al*. (2000), blastocyst quality affects the success of blastocyst stage embryo transfer. The same authors reported that fresh blastocyst transfer is a possible alternative in patients who fail to produce good quality cleavage stage embryos (Balaban *et al*., 2001). But what happens when blastocysts originating from poor cleavage stage embryos were vitrified? A second question concerns the usefulness of both vitrification and AH on blastocysts that originate from embryos showing dubious quality during their cleavage stage.

The purpose of this retrospective study is to present our clinical results obtained with vitrification of blastocysts using the HS method.

We wanted to determine to what extent the vitrification procedure induces a hardening of the ZP. We also evaluated the frequency of transfers with blastocysts in the hatching or hatched stage, the pregnancies and implantation rates after transfer of vitrified blastocysts in conjunction with an AH protocol or a non-AH protocol. Finally, we analysed the effectiveness of the HS technique according to the quality of the embryos during their cleavage stages.

Materials and methods

Patient selection

Couples entered the IVF programme for infertility treatment for male factor (ICSI with ejaculate, epididymal or testicular sperm) and/or female factor (tubal, endometriosis, idiopathic).

Fresh embryo transfers were performed after a culture period of 3 days or following culture in sequential medium for 5 days in women ranging from 27–41 years. The HS vitrification protocol was applied on supernumerary embryos that developed to the blastocyst stage after 5 days of culture.

Ovarian stimulation, oocyte retrieval, sperm preparation and embryo transfer procedures

Women undergoing assisted reproduction treatment received the combination of GnRH analogue (Suprefact, SP; Hoechst, Belgium) in association with hMG (Humegon, Organon, The Netherlands) or purified urinary FSH (Metrodin, Serono Laboratories Inc., Belgium; Menopur, Ferring, Belgium). hCG (Pregnyl, Organon; Profasi, Serono) was administered when the cohort of follicles reached a diameter of 20 mm. Luteal phase support consisted of administering 5000 IU hCG on days 4 and 8 after embryo transfer or 600 mg Utrogestan daily, vaginally, until hCG assay.

Sperm preparation was carried out using discontinuous Pure sperm (Nidacon international AB) gradient.

Oocytes were collected 34–35 h after the administration of 10 000 IU of hCG and were incubated in 0.5 ml IVF 20 medium (Scandinavian Science, AB products, Sweden). When sperm exhibited normal sperm parameters according to World Health Organization (1999) (except for morphology, for which strict Kruger criteria were used) insemination was performed after a minimal delay of 3–4 h. In cases in which patients entered the IVF programme for male or unexplained failure of fertilization, cumulus and corona cells were removed from the oocytes 2–3 h after oocyte retrieval by incubation in 25 IU/ml hyaluronidase (Type VIII; Sigma Chemical Co., USA) before performing ICSI as described previously (Vanderzwalmen *et al*., 1996).

In-vitro culture of embryos to the blastocyst stage and classification of day 3 and day 5 embryos

Embryo culture was carried out in 4-well multi-dishes (Nunc) containing each 500 µl of sequential media or in 40 µl microdrops under mineral oil (Cryo Bio System, France) at 37°C in a humidified atmosphere of 5% O_2 in air. Injected oocytes or classical inseminated oocytes were incubated in IVF 20 medium (Scandinavian Science). Sixteen to 20 h post-insemination or ICSI, all oocytes were checked for the presence of two pronuclei. After rinsing the zygotes, they were transferred into G1-2 medium (Scandinavian Science) for an additional period of 48 h.

On day 3 (66–68 h post-ICSI or insemination), the embryo cell number and the morphology were evaluated under an inverted microscope (magnification ×200). They were classified and separated in wells or micro-drops of CCM medium (Scandinavian Science) according to their quality for further culture to the blastocyst stage (day 5). The 'good' quality group consisted of grade I and II embryos. Grade I consisted of 8-cell embryos with <10% fragmentation and

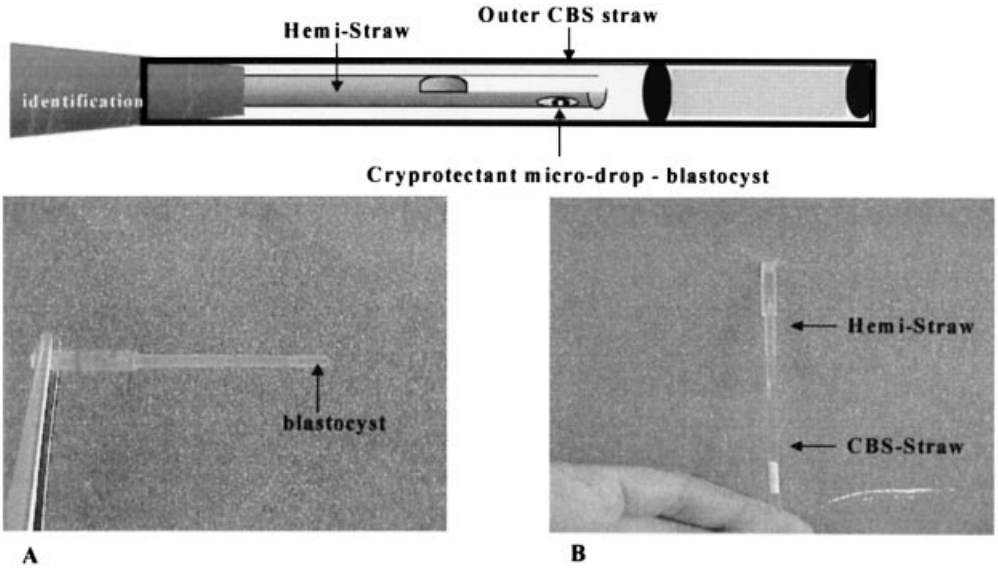

Figure 1. Scheme of the Hemi-Straw: (**A**) loading the blastocyst on the tip of the Hemi-Straw; (**B**) insertion of the Hemi-Straw into a larger 'CBS' straw.

equal-sized blastomeres. Grade II consisted of embryos with 6–8 cells, with 10–30% fragmentation and equal-sized blastomeres. The 'fair' quality group consisted of embryos with 6–8 cells, with 30–50% fragments and/or unequal-sized blastomeres. Embryos with lower morphology were not included in the study.

On day 5, the percentage of blastocyst formation was recorded and classified under an inverted microscope (magnification ×200) according to the degree of expansion of the blastocoele, the quality of the inner cell mass (ICM) and the trophectoderm (Schoolcraft *et al*., 1999).

Vitrification: preparation of the solutions

The solutions for equilibration, vitrification and thawing were prepared using Dulbecco's phosphate-buffered saline (Sigma) plus 20% human serum albumin (Irvine) (PBS–HSA).

Two solutions were prepared, one for equilibration and one for vitrification. The equilibration solution contained 10% ethylene glycol (v/v) (Fluka)–10% dimethylsulphoxide (DMSO) (v/v) (Sigma) in PBS–HSA. The vitrification solution was composed of 20% ethylene glycol (v/v)–20% DMSO (v/v) (Fluka), 25 µmol/l (10 mg/ml) Ficoll (mol. wt 400 000; Sigma) and 0.75 mol/l sucrose (Sigma) in PBS–HSA .

The solution for dilution after thawing was made of 0.5, 0.25 and 0.125 mol/l sucrose in PBS–HSA.

Vitrification of the blastocysts

Before starting the vitrification procedure, in order to reduce the negative effect of the blastocoel, expanded blastocysts were collapsed by reducing artificially with a glass pipette the volume of the blastocoele (Vanderzwalmen *et al*., 2002; Son *et al*., 2003).

Early blastocysts and shrunken blastocysts were equilibrated for 2 min at room temperature into 200 µl equilibration solution. For blastocysts, equilibration occurred at 37°C for 3 min. The blastocysts were then exposed to the vitrification solution for a maximum of 30 s. An approximate volume of 0.3 µl of cryoprotectant solution containing the blastocysts (maximum 2) was deposited using an attenuated pipette to the tip of the trough of the HS. The HS was then instantly plunged into a Dewar of LN_2 and with the aid of forceps inserted in a larger pre-cooled straw (CBS; Cryo Bio System, France) before closing it (Figure 1).

Warming procedure

The policy in our centre is to perform embryo thawing 1 day before transfer in order to assess the viability of the embryos. The warming of blastocysts occurred on day 4 post-ovulation, the transfer on day 5. Blastocyst survival post-warming was estimated by microscopic evaluation at ×200 magnification both immediately after warming and before embryo replacement (18–22 h after warming). Immediately after thawing, the vitrified blastocysts looked flat. Those showing degenerated cells were not deemed suitable for transfer. After 24 h, blastocysts exhibiting the formation of a blastocoelic cavity with preservation of structural integrity were transferred.

A Dewar of LN_2 containing the HS was placed close to the microscope. Under LN_2 and with forceps, the HS were pulled out of the larger straw and the tip of the straw holding the embryos was immediately immersed into a Petri dish containing 3 ml of 0.5 mol/l sucrose at 37°C. After 3 min, the blastocysts were transferred to 0.25 and 0.125 mol/l sucrose at intervals of 2 min at 37°C. The blastocysts were then washed several times in PBS–HSA solution, then placed in CCM medium. Afterwards, the AH technique was performed on some of the embryos.

Artificial AH procedure

Three-dimensional partial zona dissection was performed as previously described by Cieslak *et al*. (1999). After immobilization of the blastocyst on a holding pipette, the ZP was penetrated with a micro-needle (10–15 µm diameter) at the largest perivitelline space and advanced tangentially. The blastocyst was then released from the holding pipette, the micro-needle holding the embryo was gently rubbed across the holding pipette until a slice through the ZP was obtained. A large slit of approximately one-fourth of the circumference of the ZP could be obtained with the large perivitelline space. After rotating the embryo until the slit was visualized and positioned at 6 o'clock, the needle was again introduced through the opening in the perivitelline space and across the ZP perpendicular to the first one. After this procedure, a T-shaped slit was obtained.

Transfers

Approximately 20–24 h after embryo warming, 85 transfers were performed. Ongoing pregnancy was defined as the presence of gestational sac(s) with fetal heart activities at 30 days after blastocyst transfer.

Zona digestion by pronase

In order to assess if vitrification induces hardening of the ZP, the time for ZP lysis was recorded after enzymatic treatment of fresh or vitrified embryos. Zona digestion was applied on day 4 and day 5 embryos that were abnormally fertilized or that were not transferred. The ZP were enzymatically removed according to the method of Fong *et al*. (1998).

The embryos were transferred into a culture dish containing only 5 IU/ml pronase (Protease, Sigma P8811; Sigma Aldrich Co. Ltd) in CCM medium under oil. The time for complete dissolution of the zonae was recorded at ×400 magnification under an inverted Hoffman modulation contrast microscope with a 37°C stage warmer.

Statistics

χ^2-Test with Yates' correction when necessary was used to determine if differences in pregnancy rate were significant between the different categories of embryos. The percentage of blastocysts that re-expanded with or without AH was also compared using the same test. Unpaired Student's *t*-test was used to assess the statistical differences of ZP lysis times with pronase of fresh and vitrified embryos. $P < 0.05$ was considered as statistically significant.

Results

Effect of assisted hatching (Table I)

A total of 281 blastocysts were vitrified in 93 vitrification cycles with the HS technique and 274 were recovered after warming. Out of 188 (69%) embryos that survived the vitrification procedure and underwent a culture period of 20–24 h before embryo transfer, AH was performed on 82 (39 vitrification cycles) embryos.

After 24 h of culture, 72 embryos from the AH group and 96 embryos from the non-AH group developed further. As demonstrated in Table I, the overall percentages of blastocysts which hatched or were in the stage of hatching is significantly superior when the embryos had undergone AH (50%), as compared with those with non-manipulated ZP (19%) ($P < 0.001$). In the AH group, 23% of the blastocysts were expanded and 48% in the non-AH groups.

We observed a statistically significant difference in the ongoing pregnancy rate between the AH and non-AH groups. The overall pregnancy rates per vitrification–thawing cycle in the AH and non-AH groups were 38 and 19% respectively ($P < 0.05$). The implantation rate in terms of fetuses per transferred embryos were 22 and 13% for the two groups respectively ($P < 0.05$).

Table I. Vitrification–warming of blastocysts using the Hemi-Straw carrier and transfers after 24 h culture: survival and pregnancy rates in relation to assisted hatching (AH) from the zona pellucida

	AH	No AH
No. vitrification–warming cycles	39	54
No. vitrified–warmed blastocysts	119	162
No. embryos after warming	114	160
No. intact embryos after warming	82 (72)	106 (66)
No. embryos placed in culture	82	106
Embryo development after 24 h culture		
No. hatching and hatched blastocysts	41 (50)[a]	20 (19)[a]
No. expanded blastocysts	19 (23)	51 (48)
No. blastocysts–early blastocysts	12 (15)	25 (24)
No. without evolution	10 (12)	10 (9)
No. embryos surviving and transferred	72	96
No. patients with transfers	36	49
Mean blastocysts tarnsferred	2.0	2.0
Clinical pregnancy/vitrification cycles	18	13
Ongoing pregnancy/vitrification cycles	15/39 (38)[b]	10/54 (19)[b]
Implantation rates	17/72 (22)[c]	11/96 (13)[c]

Values in parentheses are percentages.
[a]$P < 0.001$; [b]$P < 0.05$; [c]$P < 0.05$.

Table II. Vitrification–warming of blastocysts using the Hemi-Straw carrier and transfers after 24 h culture: survival and pregnancy rates in relation to the quality of the embryos during their cleavage stages and with or without assisted hatching (AH)

	Embryo quality on day 3					
	Good			Fair		
	AH	No AH	Total	AH	No AH	Total
No. vitrification–warming cycles	32	39	71	7	15	22
No. embryos after warming	84	102	186	30	58	88
No. intact embryos after warming	70 (83)[a]	76 (75)[e]	146	12 (40)[a]	30 (52)[e]	42 (66)
No. embryos in culture	70	76	146	12	30	42
Embryo development after 24 h culture						
No. hatching and hatched blastocysts	37 (53)[b,h]	16 (21)[b,i]	53	4 (33)[g,h]	4 (13)[g,i]	8
No. expanded blastocysts	17 (24)	42 (55)	59	2 (17)	9 (30)	11
No. blastocysts–early blastocysts	8 (11)	15 (20)	23	4 (33)	10 (33)	14
No. without evolution	8 (11)	3 (4)	11	2 (17)	7 (23)	9
No. embryos surviving and transferred	62 (74)[c]	73 (72)[d]	135 (73)	10 (33)[c]	23 (40)[d]	33 (38)
No. patients with transfers	32	37	69	4	12	16
Mean blastocysts transferred	1.9	2.0	2.0	2.5	2.0	2.1
Clinical pregnancy/vitrification cycles	16	12	28	2	1	3
Ongoing pregnancy/vitrification cycles	13/32 (41)[f]	10/39 (27)[f]	23/71 (32)	2/7 (29)[j]	0 (0)	2/22 (9)
Implantation rates	15/62 (23) 2 twins	11/73 (16) 1 twins	26/135 (19)	2/10 (20)	0/23 (0)	2/33 (6)

[a,b,c,d]$P < 0.001$; [e]$P < 0.01$; [f]$P < 0.02$; [g,h,i]not significant.

If we consider only the pregnancy rate from the 'good' quality group, similar results in favour of the AH group are observed (Table II).

At the present time, 16 healthy babies have been born. Six children resulted from the AH group and 10 in which AH was not performed.

Correlation of day 3 embryo quality (Table II)

Independent of an intact or an open ZP, we observed that the percentage of blastocysts which survived the vitrification procedure and developed further after a period of 24 h culture correlated with the type of embryo quality observed during their development on day 3.

In all, 186 and 88 blastocysts from respectively the 'good' and 'fair' quality groups were recovered after warming. From the 'good' quality group, 73% (n = 135: 62 from AH and 73 from no AH) survived and were suitable for embryo transfers. However, only 38% (n = 33: 10 from AH and 23 from no AH) ($P < 0.001$) of embryos in the 'fair' quality group were transferable.

The percentage of blastocysts available for embryo transfer was related to previous developmental quality. In the AH group, 62 blastocysts (74%) from the 'good' group were transferred, and from the 'fair quality' group, only 10 (33%) were available for transfer ($P < 0.001$). The same trend was observed in the no-AH group where 72% of the vitrified embryos were transferred versus 40% ($P < 0.001$).

Consequently, an ongoing pregnancy rate per vitrification cycle of only 9% (2/22) was obtained in the 'fair' group. Conversely, in the 'good' quality group, an ongoing pregnancy rate of 32% (23/71) was obtained ($P < 0.05$).

Table III. Transfers after 24 h culture, ongoing pregnancies and implantation rates after transfers with at least one hatch(ing) blastocyst

	AH	No AH	Total
Total transfers	36	49	93
Transfers with at least one hatch(ing) blastocyst	25 (69)[a]	16 (33)[a]	41 (44)
Ongoing pregnancies/transfers with hatch(ing) blastocyst	12 (48)[b]	6 (38)[b]	18 (44)
Implantation rates	13/51 (25)[c]	7/25 (28)[c]	20/76 (26)

Values in parentheses are percentages.
[a]$P < 0.001$; [b,c]not significant.
AH = assisted hatching.

Table IV. Transfers after 24 h culture, ongoing pregnancies and implantation rates after transfers with at least one expanded blastocyst

	AH	No AH	Total
Total transfers	36	49	93
No. transfers with at least one expanded blastocyst	6 (17)[a]	25 (51)[a]	31 (33)
Ongoing pregnancies/transfers with expanded blastocyst	3 (50)[b]	4 (16)[b]	7 (23)
Implantation rates	3/7 (43)[c]	5/57 (9)[c]	8/64 (13)

Values in parentheses are percentages.
[a]$P < 0.001$; [c]$P < 0.05$; [b]not significant.
AH = assisted hatching.

Even though few vitrification attempts were performed, AH appears favourable in the 'fair' group considering that out of seven vitrification–thawing assays, the only two ongoing pregnancies were obtained after AH.

Percentages of transfers with expanded or hatched blastocysts after AH versus non-AH (Tables III and IV)

Table III compares the percentage of transfers performed with at least one hatching blastocyst in conjunction with an AH protocol or a non-AH protocol.

Out of 36 transfers from the AH group, 25 (69%) were performed with at least one hatching or hatched blastocyst. In the non-AH groups only 16 transfers (33%) out of the 49 were performed with at least one spontaneously hatching blastocyst ($P < 0.001$).

When at least one hatched/hatching blastocyst was transferred, no differences in the pregnancy (48% in AH group versus 38% in non-AH group) and implantation rates (25% in AH group versus 28 % in non-AH group; not significant) were observed.

Table IV compares the percentage of transfers performed with at least one expanded blastocyst in the AH and in the non-AH group.

Out of 36 transfers from the AH group, six (17%) were performed with at least one expanded blastocyst. In the non-AH group, 25 out of 49 transfers (51%) were performed with at least one expanded blastocyst ($P < 0.001$).

Higher implantation rates (43 versus 9%; $P < 0.05$) were observed in the AH group when expanded blastocysts were transferred, showing the beneficial effect of the AH procedure even if the embryos did not start the hatching process.

Enzymatic treatment of the zona pellucida

From a total of nine replicate experiments, the ZP of 18 morulae or blastocysts derived from abnormally fertilized zygotes or from blastocysts that were of insufficient quality for transfers were enzymatically dissolved after vitrification procedure or no procedure.

The enzymatic digestion of the ZP with pronase was significantly faster for the unfrozen embryos. In our working conditions, an average of 1 min 45 s was necessary to dissolve the ZP of unfrozen blastocysts and an average of 2 min 16 s for the vitrified one ($P < 0.0001$).

Discussion

The current study presents an ultra-rapid vitrification method using the HS technique. On the tip of the HS, the blastocysts are encased in a very small volume of cryoprotectant, reducing the thermo-insulating effect of a conventional straw. By dropping the tip of the HS in the thawing solution, the micro-drop is instantly warmed and the blastocysts are immediately expelled in the dilution solution reducing cytotoxic and osmotic effects. On a practical level, the use of HS vessel reduced the space required for storage inside the cryo-tank because they are placed in cryostraws instead of cryo-vials.

When compared with our classical vitrification technique inside the straw (Vanderzwalmen *et al*., 2002), the present study demonstrates that application of the HS in our assisted reproduction programme results in satisfactory and encouraging clinical results.

We observed from our preliminary vitrification attempts that the highest pregnancy rates were obtained after transfers of spontaneously hatched blastocysts as compared with transfers of intact expanded blastocysts. We therefore considered improving the pregnancy rate by artificially increasing the proportion of transfers with blastocysts in the hatching process through mechanical AH.

The beneficial effect of AH after vitrification is evident considering the significant increase in the percentage of blastocysts in hatching process and the percentage of transfers with at least one hatching or completely hatched blastocyst. Consequently, after creating a large opening in the ZP by mechanical means, our results indicate that zona-opened blastocysts implant at a significantly higher rate than their zona-intact counterparts.

When transfers were performed with mechanically or spontaneously hatched blastocysts, no significant difference was noted in the pregnancy rate (48 versus 38%) and implantation rate (25 versus 28%). Higher pregnancy rates were also observed after transfers of expanded blastocysts but with previously open ZP. In such a situation, they were classified as early blastocysts before vitrification and reached the expanded stage 24 h after warming. This indicates that, when mechanical opening is performed and/or when hatching occurred, satisfactory pregnancy and implantation rates are obtained.

The lower implantation rates observed after transfers of intact expanded blastocysts, suggests failed in-utero hatching, rendering implantation difficult or impossible.

Impaired hatching may result from ZP abnormalities, embryo quality, the effect of advanced maternal age, sub-optimal culture conditions, and possibly genetic abnormalities. It is impossible to predict after thawing which embryo will re-expand and hatch spontaneously and which will benefit from the artificial opening in the ZP.

Until now, the efficacy of AH was mainly evaluated on cleavage stage fresh embryos. There is no universal agreement regarding the efficacy of AH evaluated on fresh cleavage stage embryos and on blastocysts. However, we have concluded from our results that zona opening on vitrified embryos is useful. Therefore if cryopreservation can interfere in the spontaneous hatching process, we suggest that after vitrification an opening in the ZP or even a complete removal of the ZP after pronase may be beneficial for the clinical outcome. Tucker *et al*. (1991) and Check *et al*. (1996) showed that AH using the mechanical technique (Tucker *et al*., 1991) or the zona-drilling technique (Check *et al*., 1996) may be beneficial for frozen–thawed embryos in overcoming ZP hardening.

During the expansion of the blastocoele, the ZP becomes distended and undergoes a thinning process before rupture allowing the blastocyst to hatch. The exact nature of zona damage after freezing is not clear, the inability of cryopreserved blastocyst to hatch spontaneously may be due to changes in the structural properties of the glycoprotein membrane disturbing the elasticity and hindering the thinning process.

Different observations led us to speculate that a hardening of the ZP during vitrification may be one of the reasons that inhibit blastocysts from hatching and hence implanting. Fresh ZP are more flexible than frozen ones, leading to a difference in the resistance of the ZP when performing mechanical AH. Additionally, the resistance to enzymatic removal of the ZP by pronase increased after vitrification as compared with the unfrozen embryo. These observations suggest that hardening of the ZP *in vitro* may also be due to the cryopreservation process.

Another factor interfering in the hatching process, and in the capacity of the blastocyst to survive the vitrification process and to develop further, concerns its quality at the embryo stage. We observed that a proportion of morphologically normal-looking blastocysts that we vitrified, originated from cleavage embryos with sub-optimal morphology.

It is possible that impaired embryonic vitality may also contribute to reduced capacity of the embryo to survive vitrification and also to escape from the ZP after re-expansion.

After vitrification of blastocysts showing good quality during their development, the survival rate observed immediately after thawing, the percentage of blastocysts that hatch and re-expand after 24 h culture and the proportion of blastocysts available for embryo transfer is considerably higher as compared with those exhibiting poor embryo quality during their cleavage. Even if some blastocysts exhibit normal morphological aspects after 5 days in culture, the results of our study indicate variations in the percentage of embryos surviving immediately after thawing and that re-expanded after 24 h of culture. The post-thaw survival rates depend on the cleavage characteristics during the 5 days of culture. Only those developing from good quality cleavage stage embryos could be cryopreserved and thawed with good results. The percentage of embryos available for embryo transfer is 2-fold higher in the 'good quality' group, whereas only 40% of the vitrified 'fair' blastocysts survived the thawing and the culture period.

This underlines the need for good scoring and selection of embryos during the whole developmental stage (Alikani *et al*., 2000; Scott *et al*., 2000; Fisch *et al*., 2001). Such an approach helps to select the best embryos not only in fresh embryo transfer but also for future cryopreserved cycles. In cases of blastocysts originating from the 'fair' group, we felt that artificial hatching could be beneficial. Until now, the number of cases has been too small to demonstrate statistical differences, but we obtained only pregnancies (2/4 transfers) after performing zona hatching.

This is in agreement with Urman *et al*. (2002) who recently reported that prior to fresh embryo transfer of blastocysts, enzymatic treatment of the ZP appears to be associated with increased implantation rates, especially in patients having poor quality embryos.

In the absence of uterine enzymes, hatching is entirely dependent on embryonic vitality. This embryonic vitality corresponds to cycles of expansions and contractions of the blastocyst and to the possible secretion of lytic agents

(Gonzales *et al*., 1996). Massip *et al*. (1982) suggested in the bovine species that failure of hatching may be attributed to a functional and structural immaturity or anomaly of the trophectoderm leading to impaired accumulation and retention of fluid in the blastocoele.

Whatever the cause of failure of spontaneous hatching, the presented data provide evidence that an artificial opening created in the ZP after vitrification has no detrimental effect on the embryos and may be beneficial to overcome ZP hardening.

The only suggested adverse outcome of AH could be an increased rate of monozygotic twinning (Herschlag *et al*., 1999). At the present time, we have not observed monozygote twins after mechanical hatching procedure. This might be due to the fact that the two intersecting slits create a sizeable opening, reducing abnormal blastocyst escape (diabolo shape).

One may avoid monozygotic twinning by removing the zona pellucida completely by enzymatic treatment (Fong *et al*., 1998). We observed no adverse effect of the pronase treatment on fresh and vitrified embryos. Several pregnancies have already been obtained after soft treatment of the blastocysts before fresh or frozen embryo replacements (P.Vanderzwalmen *et al*., unpublished data).

Some groups are reluctant to culture embryos to the blastocyst stage because the freezing of supernumerary blastocysts is problematic. Our results achieved with the HS method demonstrate that human blastocysts can be successfully vitrified. From our data we conclude that morphologically good embryos resulted in blastocysts which performed better in vitrification and thawing procedures, resulting in higher implantation rates. Therefore, a good scoring and selection of embryos during the whole developmental stage is recommended.

Cryopreservation induces hardening of the ZP, which may impair spontaneous hatching after thawing. In our experience, the artificial opening of the ZP of thawed blastocysts, with the three-dimensional partial zona dissection technique, significantly improved the implantation and pregnancy rates.

The HS vitrification technique appears to be a good alternative to other carrier systems and should generate further research for vitrification of oocytes and cleavage stage embryos.

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References

Alikani, M., Caldeone, G., Tomkin, G., Garrisi, J., Koko, M. and Cohen, J. (2000) Cleavage anomalies in early embryos and survival after prolonged culture *in vitro*. *Hum. Reprod.*, **15**, 2634–2643.

Balaban, B., Urman, B., Sertac, A., Alatas, C., Aksoy, S. and Mercan, R. (2000) Blastocyst quality affects the success of blastocyst stage embryo transfer. *Fertil. Steril.*, **74**, 282–287.

Balaban, B., Urman, B., Alatas, C., Mercan, R., Aksoy, S. and Isiklar, A. (2001) Blastocyst-stage transfer of poor-quality cleavage-stage embryos results in higher implantation rates. *Fertil. Steril.*, **75**, 514–518.

Carroll, J., Depypere, H. and Matthews, C. (1990) Freeze–thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen–thawed mouse Oocytes. *J. Reprod. Fertil.*, **90**, 547–553.

Check, J., Hoover, L., Nazaria, A., O'Shaughnessy, A. and Summers, D. (1996) The effect of assisted hatching on pregnancy rates after frozen embryo transfer. *Fertil. Steril.*, **65**, 254–257.

Cieslak, J., Ivakhnenko, V., Wolf, G., Sheleg, S. and Verlinsky, Y. (1999) Three-dimensional partial zona dissection for preimplantation genetic diagnosis and assisted hatching. *Fertil. Steril.*, **71**, 308–313.

Cho, H.J., Son, W.Y., Yoon, S.H., Lee, S.W. and Lim, J.H. (2002) An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. *Hum. Reprod.*, **17**, 2419–2422.

Choi, D.H., Chung, H.M., Lim, J.M., KO, J.J., Yoon, T.K. and Cha, K.Y. (2000) Pregnancy and delivery of healthy infants developed from vitrified blastocysts in an IVF–ET program. *Fertil. Steril.*, **74**, 838–839.

De Vos, A. and Van Steirteghem, A. (2000) Zona hardening, zona drilling and assisted hatching: new achievements in assisted reproduction. *Cells Tiss. Organs*, **166**, 220–227.

Fisch, J.D., Rodriguez, H., Ross, R., Overby, G. and Sher, G. (2001) The Graduated Embryo Score (GES) predicts blastocyst formation and pregnancy rate from cleavage-stage embryos. *Hum. Reprod.*, **16**, 1970–1975.

Fong, C.Y., Bongso, A., Ng, S.C., Kumar, J., Trounson, A. and Ratman, S. (1998) Blastocyst transfer after enzymatic treatment of the zona pellucida: improving in-vitro fertilization and understanding implantation. *Hum. Reprod.*, **13**, 2926–2932.

Gonzales, D.S., Jones, J.M., Pinyopummintr, T., Carnevale, E.M. and Ginther, O.J. (1996) Trophectoderm projections: a potential means for locomotion, attachment and implantation of bovine, equine and human blastocysts. *Hum. Reprod.*, **12**, 2739–2745.

Herschlag, A., Paine, G., Cooper, W., Scholl, G.M., Rawlinson, K. and Kvapil, G. (1999) Monozygotic twinning associated with mechanical assisted hatching. *Fertil. Steril.*, **71**, 144–146.

Kuleshova, L. and Lopata, A. (2002) Vitrification can be more favourable than slow cooling. *Fertil. Steril.*, **78**, 449–454.

Lane, M., Schoolcraft, W.B. and Gardner, D.K. (1999) Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil. Steril.*, **72**, 1073–1078.

Liebermann, J. and Tucker, M.J. (2002) Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. *Reproduction*, **124**, 483–489.

Liebermann, J., Tucker, M.J., Graham, J., Han, T., Davis, A. and Levy, M. (2002) Blastocyst development after vitrification of multipronucleate zygotes using the flexipet denuding pipette (FDP). *R.B.M. Online*, **4**, 146–150.

Martino, A., Songsasen, N. and Leibo, S.P. (1996) Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. *Biol. Reprod.*, **54**, 1059–1069.

Massip, A., Mulnard, J., Vanderzwalmen, P., Hanzen, C. and Ectors, F. (1982) The behaviour of cow blastocyst in vitro: cinematographic and morphometric analysis. *J. Anat.*, **134**, 399–405.

Mukaida, T., Wada, S., Takahashi, K., Pedro, P.B., An, T.Z. and Kasai, M. (1998) Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. *Hum. Reprod.*, **13**, 2874–2879.

Mukaida, T., Nakamura, S., Tomiyama, T., Wada, S., Kasai, M. and Takahashi K. (2001) Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. *Fertil. Steril.*, **76**, 618–620.

Reed, M.L., Lane, M., Gardner, D.K., Jensen, N.L. and Thompson, J. (2002) Vitrification of human blastocysts using the cryoloop method: successful clinical application and birth of offspring. *J. Assist. Reprod. Genet.*, **6**, 304–306.

Schoolcraft, W., Gardner, D., Lane, M., Schlenker, T., Hamilton, F. and Meldrum, D. (1999) Blastocysts culture and transfer: analysis of results and parameters affecting outcome in two in vitro fertilization programs. *Fertil. Steril.*, **72**, 604–609.

Scott, L., Alvero, R., Leondires, M. and Miller, B. (2000) The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. *Hum. Reprod.*, **15**, 2394–2403.

Son, W.Y., Yoon, S.H., Yoon, H.J., Lee, S.M. and Lim, J.H. (2003) Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. *Hum. Reprod.*, **18**, 137–139

Tucker, M.J., Cohen, J., Massey, J.B., Mayer, M.P., Wiker, S.R. and Wright, G. (1991) Partial zona dissection of the zona pellucida of frozen thawed human embryos may enhance blastocyst hatching, implantation, and pregnancy rates. *Am. J. Obstet. Gynecol.*, **165**, 341–345.

Urman, B., Balaban, B., Alatas, C., Aksoy, S., Mumcu, A. and Isiklar, A.

(2002) Zona-intact versus zona-free blastocyst transfer: a prospective, randomized study. *Fertil. Steril.*, **78**, 392–396.

Vanderzwalmen, P., Bertin, G., Lejeune, B., Nijs, M., Vandamme, B. and Schoysman, R. (1996) Two essential steps for a successful intracytoplasmic sperm injection: injection of immobilized spermatozoa after rupture of the oolema. *Hum. Reprod.*, **11**, 540–547.

Vanderzwalmen, P., Delval, A., Chatziparasidou, A., Bertin, G., Ectors, F., Lejeune, B., Nijs, M., Prapas, Y., Prapas, N., Vandamme, B. *et al*. (1997) Pregnancy after vitrification of human day 5 embryos. *Hum. Reprod.*, **12** (Suppl.), 98.

Vanderzwalmen, P., Bertin, G., Debauche, C.H., Standaert, V. and Schoysman, R. (2000) In vitro survival of metaphase II oocytes and blastocysts after vitrification in an Hemi Straw (HS) system. *Fertil. Steril.*, **74** (Suppl.), 215.

Vanderzwalmen, P., Bertin, G., Debauche, CH., Standaert, V., van Roosendaal, E., Vandervorst, M., Bollen, N., Zech, H., Mukaida, T., Takahashi, K. and Schoysman, R. (2002) Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Hum. Reprod.*, **17**, 744–751.

Vatja, G., Kuwayama, M., Holm, P., Boot, P.J., Jacobsen, H., Greve, T. and Callesen, H. (1997) A new way to avoid cryoinjuries of mammalian ova and embryos: the OPS vitrification. *Mol. Reprod. Dev.*, **51**, 53–58.

World Health Organization (1999) *WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Interaction*. Cambridge University Press, Cambridge.

Yokota, Y., Sato, S., Yokota, M., Ishikawa, Y., Makita, M., Asada, T. and Araki, Y. (2000) Successful pregnancy following blastocyst vitrification. *Hum. Reprod.*, **15**, 1802–1803.

Yokota, Y., Sato, S., Yokota, M., Yokota, H. and Araki, Y. (2001) Birth of a healthy baby following vitrification of human blastocysts. *Fertil. Steril.*, **75**, 1027–1029.

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