

Relevance of assisted hatching in an oocyte donation programme using egg cryobanking: a prospective randomised study

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ABSTRACT

Objective: The objective of this prospective randomised study was to evaluate if there is an improvement in clinical outcomes when assisted hatching (AH) is performed in embryos derived from vitrified/warmed oocytes in an ovum donation programme using egg cryobanking.

Study design: Sixty oocyte recipients in a donation programme using egg cryobanking were randomly allocated to the assisted hatched (AH, $n = 30$) or control group ($n = 30$). The pregnancy and implantation rates were compared between the groups.

Results: A total of 288 vitrified oocytes were warmed for the 60 recipients. Of the 288 vitrified oocytes, 94.8% survived. All surviving oocytes were sperm injected, and 83.5% underwent fertilisation. There were 172 good-quality embryos selected for transfer. The total pregnancy rate was 40%. The pregnancy rate did not differ between the AH and control groups (43.3% and 33.3%, respectively, $p = 0.1967$), but AH resulted in a higher implantation rate (31.6% vs. 18.4%, $p = 0.0206$).

Conclusion: Our study demonstrates the effectiveness of AH in embryos derived from warmed oocytes. Our results also suggest that oocyte cryopreservation can be considered as a tool for providing highly successful outcomes in an egg donor programme.

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1. Introduction

The cryopreservation of oocytes enables patients who are at risk of losing ovarian function because of gonadotoxic treatment or surgery to store their oocytes for later use. Egg banking also provides women with the option to delay their childbearing and circumvents the ethical concerns that are associated with embryo preservation [1].

Currently, ovum donation is a well-established and successful practice that is commonly applied as a response to many clinical situations and leads to the highest pregnancy rates reported for any assisted reproductive method [2].

The clinical outcome of oocyte donation depends upon the presence of a receptive endometrium, which is usually prepared by exogenous hormone replacement [3,4], and the well-synchronised replacement of good-quality embryos. For the synchronisation, several strategies have been employed with varying levels of success, but the most crucial factor is certainly the availability of

oocyte banks. The establishment of banks of donated oocytes has considerably simplified the logistics and means by which oocytes could be donated.

In spite of the numerous studies conducted over the past 20 years, the reliability of oocyte cryopreservation has only recently been confirmed. Ever since the first report of a live birth after oocyte cryopreservation [5], the slow cooling method has been applied with varying levels of success [3,6–9].

Novel approaches to slow freezing have been introduced that have resulted in improved oocyte survival rates and subsequent embryo development. As an alternative to slow freezing, the vitrification procedure has recently been applied to human oocytes and embryos [10,11]. This procedure uses very high cooling rates and high concentrations of cryoprotectants, in which a solution or specimen solidifies to form a glass-like, or vitreous, structure without any ice crystal formation during cooling.

The vitrification method has resulted in a significant improvement in post-thaw/warming cell survival and viability, subsequent fertilisation, embryo development and clinical outcomes [11–15]. Despite such enhancements, however, the highest reported pregnancy rate achieved per cryopreserved oocyte after fertilisation and embryo transfer has been relatively low [3].

The difficulties associated with oocyte cryopreservation are primarily related to the special structure and sensitivity of this cell,

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the time of exposure to the cryoprotectant solutions, the concentration of the cryoprotectants, the equilibrium temperature and the extra- and intra-cellular ice formation, which affect the viability and physiology of oocytes [16]. Moreover, the hardening of the zona pellucida of the cryopreserved oocytes was also shown to be increased by the freeze–thaw process [17].

Although the zona pellucida has multiple functions during oocyte and embryo development, the hatching of the blastocyst from the zona pellucida is a prerequisite for implantation in the uterus. In fact, it has been demonstrated that the reduced implantation ability observed following *in vitro* fertilisation (IVF) and embryo transfer may be caused by the inability of the embryo to hatch out of the zona pellucida [18].

In the past decade, several techniques for assisted hatching (AH) in IVF programmes have been introduced to breach or thin the zona pellucida and assist in the natural process of hatching. Few prospective, randomised studies, however, have specifically addressed the issue of AH performed on frozen-thawed embryos during IVF cycles, and previous studies have reported controversial results with decreased [19], similar [20] or increased [21] implantation rates in the AH group as compared to the control group. Moreover, there is no available literature supporting the relevance of AH in embryos derived from vitrified/warmed oocytes.

The goal of this prospective, randomised trial was to verify if there is an improvement in clinical outcomes when AH is performed in embryos derived from vitrified/warmed oocytes in an ovum donation programme using egg cryobanking.

2. Materials and methods

2.1. Study design

Sixty oocyte recipients in a donation programme using egg cryobanking were randomly allocated to the assisted hatched (AH, $n = 30$) or control group ($n = 30$). The patients' randomisation was carried out using the SAS System for Windows and was performed just before embryo transfer by the IVF laboratory staff. The pregnancy and implantation rates were compared between the groups.

Pregnancy was defined as the presence of foetal heart activity on ultrasound at six to seven weeks of gestation, and implantation was defined as the presence of gestational sac visualised on ultrasound at four to six weeks after embryo transfer.

A written informed consent was obtained, in which patients agreed to share the outcomes of their own cycles for research purposes, and the study was approved by the local institutional review board.

2.2. Preparation of oocytes and vitrification/warming process

The retrieved oocytes were maintained in human tubal culture medium (HTF, Irvine Scientific, Santa Ana, USA) supplemented with 10% Human Synthetic Albumin (HSA, Irvine Scientific, Santa Ana, USA) and covered with mineral oil (Ovoil™, Vitrolife) for 2–4 h before cumulus cell removal. The surrounding cumulus cells were removed after exposure to a HEPES-buffered medium containing hyaluronidase (80 IU/mL, Irvine Scientific, Santa Ana, USA). The remaining cumulus cells were then mechanically removed by gently pipetting with a hand-drawn Pasteur pipette (Humagen Fertility Diagnostics, Charlottesville, USA).

The denuded oocytes were then assessed for nuclear status. Oocytes that were observed to have released the first polar body were considered to be mature and were vitrified 3 h after collection and cryo-stored. The vitrification and warming procedures were performed using the Cryotop method [13].

2.3. Intracytoplasmic sperm injection and laser-assisted hatching

Intracytoplasmic sperm injection (ICSI) was performed 2 h after warming in a micro-injection dish that had been prepared with 4 μ L droplets of buffered medium (HEPES, Irvine Scientific, Santa Ana, USA), covered with mineral oil and placed on a heated stage (37.0 ± 0.5 °C) of an inverted microscope. Approximately 16 h after the ICSI, fertilisation was confirmed by the presence of two pronuclei and the extrusion of the second polar body. The embryos were kept in a 50 μ L drop of HTF medium supplemented with 10% HSA and were covered with mineral oil in a humidified atmosphere under 6% CO₂ at 37 °C for three days.

Immediately before embryo transfer, the embryos undergoing laser-assisted hatching had the zona pellucida drilled by making a small (30 μ m) opening away from the edges of the holes using a 1.48 μ m wavelength diode laser (Fertilase, OCTAX Laser Shot™, Herbron Germany) under an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon®, Tokyo, Japan).

2.4. Statistical analysis

Patient randomisation was carried out using the SAS System for Windows (PROC PLAN, seed = 1234) Statistical Program.

The mean values were compared using a Student's *t* or Mann–Whitney test as appropriate, and proportions were compared using the chi-squared test. The results are expressed as the mean \pm standard deviation (SD) for numeric variables and percentages (%) for categorical variables.

To study the influence of AH on pregnancy outcome, a binary logistic regression was performed, and to study the influence of AH on implantation, a linear logistic regression was conducted. All regression analyses were adjusted for maternal age, the number of retrieved oocytes, endometrial thickness, and fertilisation rate, as these would be considered to be potential confounders of the association between the factor evaluated and the ICSI outcomes.

The results are expressed as either odds ratios (OR) (with 95% confidential intervals (CI)) or regression coefficients (RC). *P* values are also reported. The results were considered to be significant at the 5% critical level ($p < 0.05$). The data analysis was carried out using the Minitab (version 14) Statistical Software.

3. Results

A total of 288 vitrified MII oocytes were warmed for the 60 recipients (4.8 oocytes per recipient). Of the 288 vitrified oocytes, 273 (94.8%) survived. All surviving oocytes underwent ICSI, and 228 achieved normal fertilisation (83.5%). There were 172 good-quality embryos selected for transfer (75.4%), and 43 of these implanted (25.0%, Fig. 1). The implantation rate per warmed oocyte was 14.9%.

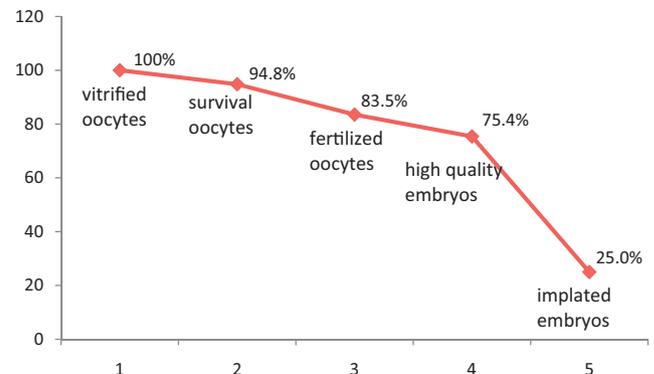


Fig. 1. Graphic illustration of the percentage of vitrified oocytes, surviving oocytes, fertilised oocytes, high-quality embryos and implanted embryos.

Table 1

Characteristics of patients for whom embryos were or were not submitted to assisted hatching.

Groups	Control	Assisted hatching	P
n	30	30	
Female age	37.1 ± 3.1	37.6 ± 2.7	0.201
Body mass index	22.3 ± 2.8	22.3 ± 3.4	0.854
Fertilisation rate	85.5%	83.6%	0.324
Rate of high-quality embryos	74.9%	75.1%	0.789
Embryos transferred	2.2 ± 0.9	2.1 ± 1.0	0.479
Endometrium thickness	10.8 ± 1.8	10.6 ± 1.7	0.482
Pregnancy rate	10/30 (33.3%)	13/30 (43.3%)	0.196
Implantation rate	18.4%	31.6%	0.020

Mann–Whitney and Chi-squared tests.

The demographic and cycle characteristics were not significantly different between the groups (Table 1).

A total of 24 patients achieved clinical pregnancy after vitrification/warming (total pregnancy rate of 40%). The clinical pregnancy rate did not differ between the AH and control groups (43.3% and 33.3%, respectively, $p = 0.1967$, Table 1), but AH resulted in a significantly higher implantation rate (31.6% vs. 18.4%, $p = 0.0206$, Table 1).

These findings were confirmed by regression models for pregnancy (OR = 1.14; IC 95% = 0.80–1.72; $p = 0.766$) and for implantation rate (RC: 19.45, $P = 0.041$).

4. Comment

The success rate of IVF/ICSI is dependent upon a number of factors that have been optimised to increase pregnancy rates. Although high-quality embryos are available for transfer, however, only a small proportion of patients undergoing IVF/ICSI will ever achieve a pregnancy. In fact, it has been reported that up to 80% of the embryos placed into the uterine cavity fail to implant [22]. This may be attributed to multiple factors, including embryo anomalies, endometrial receptivity and hatching failure [23]. The successful hatching of embryos is thought to be a key event in the implantation process. If the hatching does not take place, further embryo development will not occur.

It has been reported that the cryopreservation thawing procedure may impact an embryo's implantation potential by hardening the zona pellucida [18]. Therefore, assisted zona hatching was introduced in IVF programmes to breach the zona pellucida and assist in the natural process of hatching [24]. The present study investigated the contribution of laser AH in embryos derived from vitrified/warmed oocytes, and a positive effect on the embryo implantation potential was noted.

Assisted hatching can be done by mechanical, chemical or laser manipulation, but the laser appears to be associated with a better outcome than that of the chemical [25] or mechanical [26] methods. Although assisted zona hatching had been introduced into IVF programmes to breach the zona pellucida and to assist in the natural process of hatching [24], the importance of zona pellucida integrity in cleavage stage embryos has been highlighted. Yano et al. [27] indicated a higher pregnancy rate after the transfer of partial zona pellucida-thinned embryos than with circumferential zona pellucida-thinned embryos.

In fact, the zona pellucida is extremely important for oocyte and embryo development. Before fertilisation, it presents a species-specific sperm barrier, and immediately following fertilisation, it acts as a block to polyspermy [28]. It also protects the embryo against infiltration by leukocytes and microorganisms (5) in the reproductive tract. Moreover, it maintains the cell arrangement of early embryos to ensure their successful development [29].

While some authors have reported the benefits of AH in frozen/thawed embryos [21,30,31], others have failed to detect the same

effect [20,32]. In addition, the literature regarding the effects of AH in embryos derived from the vitrified/warming of oocytes is quite scarce.

Oocyte freezing may offer the possibility of extending the reproductive capability of young women with malignant diseases in cases where treatment involving surgery, chemotherapy or radiotherapy may compromise the ovarian reserve [33]. Cryopreservation can also offer alternatives for infertile patients who are subject to ovarian hyper-stimulation syndrome, those who are poor responders to ovarian stimulation and women who are undergoing premature ovarian failure [34]. Moreover, human oocyte cryopreservation solves the legal restrictions and ethical problems that are associated with the cryopreservation of embryos in patients undergoing IVF [35].

Additionally, successful oocyte freezing/thawing technology would potentially aid donation programmes [36], which are currently strongly limited in their efficiency by different factors, including the availability of qualifying donors, expense, delay in synchronisation, and ethical concerns regarding supernumerary embryo disposition. An oocyte cryo-bank would provide a more efficient sharing of donor eggs between multiple recipients, improving cost effectiveness through the better use of resources.

Clearly, the most crucial prerequisite for a successful egg cryo-banking programme is to have an efficient oocyte freezing/thawing technology. In spite of its evident clinical relevance, however, the development of efficient cryopreservation techniques has represented a real challenge, as both freezing and thawing exposes cells to severe stress and can potentially cause cell damage. Oocyte cryopreservation by vitrification, however, represents a feasible alternative that has been producing satisfactory outcomes [11,37].

These gametes must withstand the vitrification/warming process to generate competent embryos that are capable of implanting. Along this complex pathway, they must overcome all the effects of the physicochemical changes that occur during equilibrium and vitrification, successfully survive the storage period and finally return to physiological temperatures. Although it is well known that several factors may influence this intricate process, in the present study, satisfactory survival, fertilisation and implantation rates were observed for warmed oocytes. Moreover, concerns relating to the hardening of the zona pellucida were alleviated, as demonstrated by the higher implantation rate observed when AH was performed.

Our rates are consistent with other studies that have reported improved survival, implantation, and pregnancy rates when vitrification was used instead of the slow freezing method [12,14,38,39]. Together with the studies cited above, the results of the current study support the concept that oocyte cryopreservation can be performed successfully in a routine IVF programme, providing the foundation for the employment of egg cryobanking, especially in donor-recipient programmes.

Nagy et al. [11] compared the results of fresh and frozen oocyte donation cycles and showed that the number of oocytes allocated per recipient was approximately five times higher in fresh cycles. The number of warmed oocytes per recipient was relatively low as a consequence of the high survival rates, confirming previous observations of vitrification. In fact, as many or most recipients end up having many more embryos in a fresh donation cycle than they would ever possibly use, most of them remain frozen, adding to the ever-increasing problem of cryo-stored embryos.

In the present study, the implantation rate, calculated based on the number of oocytes warmed, reached nearly 15.0%. In other words, approximately six warmed oocytes resulted in one implantation. This favourable outcome confirms the potential of oocyte vitrification and suggests that the efficiency of oocyte donation can be significantly improved using cryo-banking. Oocyte cryo-banking may also reduce frozen embryo storage and, as

described before, may help with donor-recipient synchronisation and possibly reduce costs.

In summary, our results demonstrate the effectiveness of AH in embryos derived from warmed oocytes in a donation programme using egg-cryobanking. These results further suggest that oocyte cryopreservation can be considered as a tool to provide highly successful outcomes in an egg donor programme and encourage the use of oocyte cryo-banking for egg donation purposes.

References

- [1] Koutlaki N, Schoepper B, Maroulis G, Diedrich K, Al-Hasani S. Human oocyte cryopreservation: past, present and future. *Reproductive Biomedicine Online* 2006;13:427–36.
- [2] Sauer MV, Kavac SM. Oocyte and embryo donation 2006: reviewing two decades of innovation and controversy. *Reproductive Biomedicine Online* 2006;12:153–62.
- [3] Nottola SA, Macchiarelli G, Cotichio G, et al. Ultrastructure of human mature oocytes after slow cooling cryopreservation using different sucrose concentrations. *Human Reproduction* 2007;22:1123–33.
- [4] Pados G, Camus M, Van Steirteghem A, Bonduelle M, Devroey P. The evolution and outcome of pregnancies from oocyte donation. *Human Reproduction* 1994;9:538–42.
- [5] Cheng H, Shang X, He Y, Zhang T, Zhang YP, Zhou R. Insight into human sex ratio imbalance: the more boys born, the more infertile men. *Reproductive Biomedicine Online* 2007;15:487–94.
- [6] Fabbri R, Porcu E, Marsella T, Rocchetta G, Venturoli S, Flamigni C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. *Human Reproduction* 2001;16:411–6.
- [7] Levi Setti PE, Albani E, Novara PV, Cesana A, Morreale G. Cryopreservation of supernumerary oocytes in IVF/ICSI cycles. *Human Reproduction* 2006;21:370–5.
- [8] Allan J. Re: Case report: Pregnancy from intracytoplasmic injection of a frozen-thawed oocyte. *The Australian & New Zealand Journal of Obstetrics & Gynaecology* 2004;44:588.
- [9] Oktay K, Cil AP, Bang H. Efficiency of oocyte cryopreservation: a meta-analysis. *Fertility and Sterility* 2006;86:70–80.
- [10] Kupker W, Al-Hasani S, Schulze W, et al. Morphology in intracytoplasmic sperm injection: preliminary results. *Journal of Assisted Reproduction and Genetics* 1995;12:620–6.
- [11] Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reproductive Biomedicine Online* 2006;12:779–96.
- [12] Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reproductive Biomedicine Online* 2005;11:300–8.
- [13] Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology* 2007;67:73–80.
- [14] Lucena E, Bernal DP, Lucena C, Rojas A, Moran A, Lucena A. Successful ongoing pregnancies after vitrification of oocytes. *Fertility and Sterility* 2006;85:108–11.
- [15] Chian RC, Huang JY, Tan SL, et al. Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes. *Reproductive Biomedicine Online* 2008;16:608–10.
- [16] Gardner DK, Sheehan CB, Rienzi L, Katz-Jaffe M, Larman MG. Analysis of oocyte physiology to improve cryopreservation procedures. *Theriogenology* 2007;67:64–72.
- [17] Manna C, Rienzi L, Greco E, et al. Zona pellucida solubility and cortical granule complements in human oocytes following assisted reproductive techniques. *Zygote* 2001;9:201–10.
- [18] Cohen J, Elsner C, Kort H, et al. Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. *Human Reproduction* 1990;5:7–13.
- [19] Primi MP, Senn A, Montag M, et al. A European multicentre prospective randomized study to assess the use of assisted hatching with a diode laser and the benefit of an immunosuppressive/antibiotic treatment in different patient populations. *Human Reproduction* 2004;19:2325–33.
- [20] Ng EH, Naveed F, Lau EY, et al. A randomized double-blind controlled study of the efficacy of laser-assisted hatching on implantation and pregnancy rates of frozen-thawed embryo transfer at the cleavage stage. *Human Reproduction* 2005;20:979–85.
- [21] Gabrielsen A, Agerholm I, Toft B, et al. Assisted hatching improves implantation rates on cryopreserved-thawed embryos. A randomized prospective study. *Human Reproduction* 2004;19:2258–62.
- [22] Ashwood-Smith MJ, Edwards RG. DNA repair by oocytes. *Molecular Human Reproduction* 1996;2:46–51.
- [23] Mantoudis E, Podsiadly BT, Gorgy A, Venkat G, Craft IL. A comparison between quarter, partial and total laser assisted hatching in selected infertility patients. *Human Reproduction* 2001;16:2182–6.
- [24] Cohen J, Alikani M, Trowbridge J, Rosenwaks Z. Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. *Human Reproduction* 1992;7:685–91.
- [25] Hsieh YY, Huang CC, Cheng TC, Chang CC, Tsai HD, Lee MS. Laser-assisted hatching of embryos is better than the chemical method for enhancing the pregnancy rate in women with advanced age. *Fertility and Sterility* 2002;78:179–82.
- [26] Makrakis E, Angeli I, Agapitou K, Pappas K, Dafereras A, Pantos K. Laser versus mechanical assisted hatching: a prospective study of clinical outcomes. *Fertility and Sterility* 2006;86:1596–600.
- [27] Yano K, Yano C, Kubo T, Ohashi I, Maeda N, Fukaya T. Chemical zona thinning with acidified Tyrode's solution: comparison between partial and circumferential techniques. *Journal of Assisted Reproduction and Genetics* 2007; 24:471–5.
- [28] Wassarman PM. Mouse gamete adhesion molecules. *Biology of Reproduction* 1992;46:186–91.
- [29] Suzuki H, Togashi M, Adachi J, Toyoda Y. Developmental ability of zona-free mouse embryos is influenced by cell association at the 4-cell stage. *Biology of Reproduction* 1995;53:78–83.
- [30] Ng EH, Lau EY, Yeung WS, Cheung TM, Tang OS, Ho PC. Randomized double-blind comparison of laser zona thinning and breaching in frozen-thawed embryo transfer at the cleavage stage. *Fertility and Sterility* 2008; 89:1147–53.
- [31] Valojerdi MR, Eftekhari-Yazdi P, Karimian L, Ashtiani SK. Effect of laser zona thinning on clinical outcome of assisted reproduction technology in patients with advanced female age, recurrent implantation failure, or frozen-thawed embryos. *Fertility and Sterility* 2008;90:84–91.
- [32] Kacem O, Sifer C, Barraud-Lange V, et al. Sperm nuclear vacuoles, as assessed by motile sperm organellar morphological examination, are mostly of acrosomal origin. *Reproductive Biomedicine Online* 2011;20:132–7.
- [33] Yang D, Brown SE, Nguyen K, Reddy V, Brubaker C, Winslow KL. Live birth after the transfer of human embryos developed from cryopreserved oocytes harvested before cancer treatment. *Fertility and Sterility* 2007;87: 1469 e1–e4.
- [34] Li XH, Chen SU, Zhang X, et al. Cryopreserved oocytes of infertile couples undergoing assisted reproductive technology could be an important source of oocyte donation: a clinical report of successful pregnancies. *Human Reproduction* 2005;20:3390–4.
- [35] Parmegiani L, Cognigni GE, Bernardi S, et al. Freezing within 2 h from oocyte retrieval increases the efficiency of human oocyte cryopreservation when using a slow freezing/rapid thawing protocol with high sucrose concentration. *Human Reproduction* 2008;23:1771–7.
- [36] Tucker MJ, Morton PC, Wright G, Sweitzer CL, Massey JB. Clinical application of human egg cryopreservation. *Human Reproduction* 1998;13:3156–9.
- [37] Grifo JA, Noyes N. Delivery rate using cryopreserved oocytes is comparable to conventional in vitro fertilization using fresh oocytes: potential fertility preservation for female cancer patients. *Fertility and Sterility* 2010;93:391–6.
- [38] Antinori M, Licata E, Dani G, et al. Intracytoplasmic morphologically selected sperm injection: a prospective randomized trial. *Reproductive Biomedicine Online* 2008;16:835–41.
- [39] Cobo A, Kuwayama M, Perez S, Ruiz A, Pellicer A, Remohi J. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. *Fertility and Sterility* 2008;89:1657–64.