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COMBINED ARTIFICIAL COLLAPSE AND ASSISTED HATCHING INCREASE THE SUCCESS RATE IN FROZEN EMBRYO TRANSFER

Lyuboslava Valkova#, Tanya Milachich, Tanya Timeva, Atanas Shterev

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Abstract

Human embryo cryopreservation is a contemporary procedure, widely used in assisted reproductive technology. It is of exceptional importance to assess all existing methods for improving embryo survival rate and increasing pregnancy and live birth rates. In the present study we examine the effects of artificial collapse on the survival rate of vitrified blastocysts and the impact of assisted hatching on the clinical pregnancy rate when frozen embryo transfer is performed. In addition we provide statistically significant data demonstrating the beneficial effect of combination of these procedures on the success rate for pregnancy after frozen embryo transfer. Embryologists might benefit from the approaches presented in this study in order to improve assisted reproductive technology outcomes.

Key words: vitrfication, blastocyst, artificial collapse, assisted hatching

Introduction. Cryopreservation of human embryos maximizes the effectiveness of the in vitro fertilization (IVF) cycle and thus has become a preferred choice and a routine procedure in human assisted reproduction [1]. Among different methods for embryo freezing, vitrification is favoured because it eliminates the possibility of formation of damaging ice crystals. This is achieved by very fast cooling, ranging between 20 000 to 100 000 °C/min in small volumes and in the

#Corresponding author.
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presence of high concentrations of cryoprotectants [2,3]. Despite the documented advantages, vitrification may be hampered or may induce unfavourable changes in the embryo that can be avoided by additional embryo manipulations benefiting its survival and implantation.

Freezing could be done on cleavage-stage embryos or blastocysts but vitrification of blastocysts (day 5 embryos) is recommended because they have higher implantation potential. Important feature of blastocysts is the presence of blastocoel, filled with fluid that may interfere with freezing by forming ice crystals, lethal for the blastocysts. Removal of the blastocoel fluid could decrease the risk of injury of the cells. The blastocoel is observed to collapse spontaneously in response to major physicochemical change in the environment but it can also be artificially induced. This intentional removal of the fluid from the blastocoel, called artificial collapse (AC) has been reported to improve survival rate and to increase the clinical success of blastocyst cryopreservation programmes [4].

Cryopreservation could also damage the elasticity of the glycoprotein layer surrounding the plasma membrane of mammalian oocytes, termed zona pellucida (z.p.) by inducing its "hardening" [5,6]. Zona pellucida is necessary during the first days of embryo development but the embryo has to leave it in order to implant in the uterus. This is a natural process, known as blastocyst hatching that includes thinning and tearing of z.p. through enzymatic activity [7] and repeated expansions and contractions of the blastocyst [8]. Vitrification-induced hardening of z.p. could delay hatching which may possibly account for the reduced fertility rates of vitrified embryos. Assisted hatching (AH) is a laboratory procedure involving artificial thinning or drilling of zona pellucida which favours embryo hatching. There are three techniques for performing AH: mechanical, chemical and laser-mediated. Laser represents the best tool for microsurgical procedures and thus nowadays assisted hatching by laser (AHL) is the method of choice.

Cryopreserved embryos are used for frozen embryo transfer (FET) after warming. Here we test the hypothesis that combining assisted hatching and artificial collapse of blastocysts may significantly increase the success rate in FET.

Materials and methods. ***Sample collection.*** All patients included in the study were treated for infertility by controlled ovarian hyperstimulation with gonadotropins finished with oocyte pick up. Classical in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) was performed to the retrieved oocytes. All obtained (in case of freeze all strategy) or surplus (after fresh ET) embryos on day 5 (blastocysts) with good quality were cryopreserved by vitrification. Before vitrification all patients signed informed consent about cryostorage, embryo manipulations and following FET. After thawing, additional informed consent, concerning assisted hatching by laser, was signed.

Embryo vitrification. Analyzed preimplantation human embryos on blastocyst stage were vitrified using VitKit Freeze (Irvine, USA) according to the manufacturer instructions. They were stored in liquid nitrogen ($-196\,^{\circ}$C) in De-

war tanks and were warmed for FET by VitKit Thaw (Irvine, USA).

Artificial collapse. AC were performed in buffered media for embryo manipulation outside incubator GMOPS (Vitrolife, Sweden) by two different methods – by injecting micropipette (MicroTech, Czech Republic) and by laser (Zona Infrared Laser Optical System, ZILOS; Hamilton-Thorne Research, Beverly, MA, USA, with 1.48-mm infrared diode laser beam positioned on an inverted microscope (Axio Observer 1, Carl Zeiss). The micropipette was used to extract blastocoel fluid after puncturing blastocyst, away from the inner cell mass (ICM). When laser was used, two or three beams were applied on the trophoblast cells.

Frozen embryo transfer. The embryos were warmed and cultured 2 hours before the transfer into the uterine cavity by special catheter. The endometrium of the uterus was hormonally prepared by estradiol and progesterone. After FET, subsequent luteal support by progesterone was performed.

Assisted hatching. AH by laser was performed half an hour after warming when blastocysts were still not re-expanded and there was no risk for injury of the ICM. A hole in zona pellucida was made by two to five, 0.5 ms laser pulses by inverted microscope (Axio Observer 1, Carl Zeiss) with Zona Infrared Laser Optical System with 1.48-mm infrared diode laser beam.

Statistics. For statistical analysis the computer program MedCalc, version 16.2 with "N-1" Chi-squared test was used. $P < 0.05$ was considered statistically significant.

Results and discussion. Artificial collapse is the release of excessive amount of fluid in the blastocoel in order to obtain a better survival of blastocysts after vitrification. It can be induced by a number of treatments like laser beam, by taking out the fluid with micropipette, pipetting the blastocyst multiple times with tight pipette and also incubation in hyperosmotic solution of sucrose [9,10]. Since there are only a few studies, comparing the effectiveness of the different methods [9,11] we studied 67 blastocysts, collapsed by two different methods. The first group included 34 blastocysts, collapsed by the use of ICSI pipette and the second group, consisted of 33 blastocysts that were treated by laser. Independent of the type of treatment the blastocoel fluid was successfully removed (Fig. 1) and embryos were vitrified.

Calculating the survival rate after thawing, we found that both techniques produced high percentage of viable blastocysts – 82.3% for AC performed with ICSI pipette and 81.8% for laser-induced AC with no statistically significant difference ($P = 0.96$) between the two methods. These results justified the use of both techniques in all of the following procedures.

Removing the blastocoel fluid from blastocysts before vitrification is not a commonly used technique. Some studies do not find improvement in survival and clinical pregnancy rates [12] when AC vitrified embryos are used, while others have come to the opposite conclusions [4,11,13]. This discrepancy determined our next studies. We analyzed 937 blastocysts of which 452 were without AC and 485

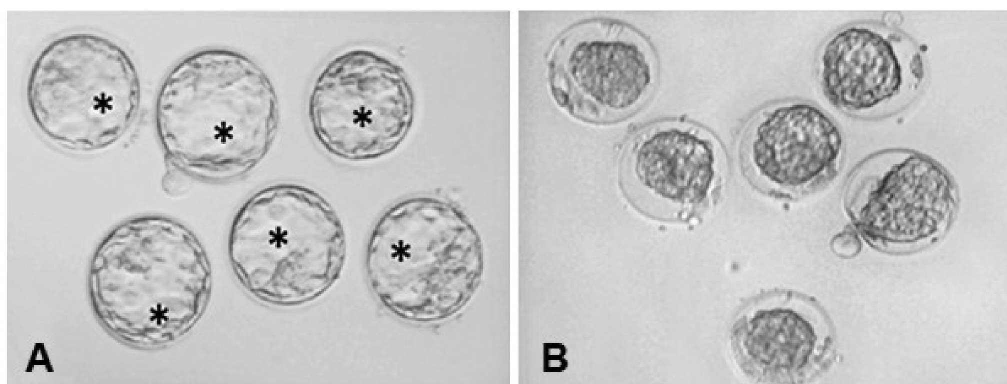

Fig. 1. Representative images of artificial collapse before blastocyst vitrification. A) Blastocyst before removal of blastocoel fluid; B) Collapsed blastocyst after artificial collapse. Star (*) indicates the blastocoel

were artificially collapsed by micropipette or laser methods. Our results convincingly demonstrated (Table 1) that applying AC resulted in statistically significant increase ($P = 0.007$) in the survival rate of the blastocysts after usage of AC (85.2%) compared to the group that were not subjected to AC (78.1%).

Table 1

Effect of AC on the survival rate of vitrified blastocysts

Treatment	Blastocysts (n)	Survival rate (n)	Survival rate (%)	P value
Without AC	452	353	78.10%	$P = 0.007$
With AC	485	413	85.20%	

As it has been suggested by Villami et al. [14] the improved survival rate of blastocysts after AC may come not only from the removal of the excessive liquid but in addition it may be due to the hole formation in z.p. which ensures faster penetration of cryoprotectants into the blastocysts.

While zona pellucida is important for protection of the embryo in the very early stages of development, at the expanded blastocyst stage it has to be removed in order for implantation to take place. Failure of z.p. to rupture and subsequent compromised hatching have been suggested to reduce the implantation rates of assisted reproductive techniques [15, 16]. Therefore, assisted hatching should be regarded as a method for improving implantation by allowing earlier embryo–endometrium contact. A typical result of laser-induced hatching is presented in Fig. 2.

It is the most effective and harmless for the embryo technique compared to chemical and mechanical AH. Our unpublished data found that laser drilling of z.p. leads to higher pregnancy rate compared to thinning. These data support

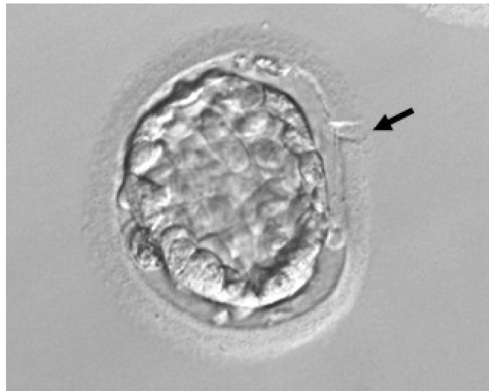

Fig. 2. Representative image of blastocyst on day five, after drilling of zona pellucida by laser. The arrow indicates the hole made in the z.p.

the finding of SCHIMMEL et al. [17] who demonstrated that zona opening helps the process of hatching of mice embryos unlike zona thinning. In all samples, we introduced a hole in z.p., instead of thinning it.

There are a number of studies on the effect of AH but most of them are using embryos on day two [18] or day three [15,19]. Although most of the studies find positive correlation between AH and pregnancy rate [16,20] this method is still not used routinely in IVF practice.

We studied the effect of assisted hatching induced by laser (AHL) in 112 FET. Forty-seven FET procedures were without AHL, and 65 were performed after applying AHL. The obtained clinical pregnancy rates were 25.5% and 56.9%, respectively. The results demonstrated statistically significant (= 0.0009), more than double increase of the clinical pregnancy rate when compared to the rate, obtained for embryos without AH.

Since our results demonstrated that each of the studied techniques – AC and AH have beneficial effects on critical steps of the frozen embryo transfer procedure we were interested to test whether a combination of these methods will be advantageous for the FET outcome. We analyzed 251 FET with blastocysts, all of which were subjected to AHL. They were divided into two groups – the first one included 129 FET without AC of blastocysts and the second one consisted of 122 FET, performed after AC. In agreement with our previous results, the cancellation rate, which is related to the survival rate after thawing of vitrified blastocysts was much higher in the first group (11.6%) while only 2.5% of the AC blastocysts did not survive vitrification and FET procedure was cancelled (Table 2). The same was true for the main parameter of interest. The clinical pregnancy rate, obtained when AC/AH blastocysts were used (41.2%), demonstrated statistically significant increase (P=0.02) when compared with FET blastocysts that were not

collapsed before vitrification (26.3%).

Table 2

Effect of combined AC and AH on clinical pregnancy rate of IVF procedures

Treatment	FET(n)	Performed FET (n)	Clinical pregnancy rate (n) and (%)	P value pregnancy rate	Cancellation rate (n) and (%)	P value cancellation rate
without AC/ with AH	129	114	30 (26.3%)	P=0.02	15 (11.6%)	P=0.005
with AC/ with AH	122	119	49 (41.2%)		3 (2.5%)	

Our results support the raising notion that a combination of AC and AH is beneficial for the FET performed with blastocysts and has to be applied routinely in IVF procedures.

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In Vitro Fertilization Unit
Obstetric Gynecological Hospital Dr Shterev
25–31 Hristo Blagoev St
1330 Sofia, Bulgaria
e-mail: petkoval@yahoo.com
tanya_ivf@yahoo.com
ttimeva@yahoo.com
ashterev@gmail.com