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Artificial blastocoel collapse of human blastocysts before vitrification and its effect on re-expansion after warming – a prospective observational study using time-lapse microscopy

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Borut Kovačič was awarded a PhD by the University of Ljubljana, in the field of the molecular aspects of fertilization failures after ICSI. He was involved in several research projects on male infertility treatment, natural cycles in human IVF and optimization of blastocyst culture and cryopreservation. Today he is the Head of the IVF Laboratory in Maribor and Associate Professor in Cell Biology at the University of Ljubljana.

KEY MESSAGE

Human blastocysts that were artificially collapsed before vitrification re-expanded more rapidly and by a slightly different pattern after warming compared with their intact counterparts. Limited data suggest that these new parameters are not predictive of live birth, but this remains to be further investigated.

A B S T R A C T

Vitrified human blastocysts show varied re-expansion capacity after warming. This prospective observational study compared behaviour of artificially collapsed blastocysts (study group patients, $n = 69$) to that of blastocysts that were vitrified without artificial collapse (control group patients, $n = 72$). Warmed blastocysts were monitored by time-lapse microscopy and blastocoel re-expansion speed and growth patterns compared between study and control groups. These parameters were also retrospectively compared between blastocysts that resulted in live birth and those that failed. Artificially collapsed blastocysts re-expanded on average 15.01 μm^2/min faster than control blastocysts ($P = 0.0013$). Warmed blastocysts expressed four different patterns of blastocoel growth. The pattern showing contractions at the end of culture was observed to have a lower prevalence in control blastocysts, which coincided with the lower incidence of hatching in this group. Re-expansion speed and prevalence of growth patterns were comparable between blastocysts that did and did not result in a live birth. This was seen in the study and control groups. Despite faster re-expansion and different growth patterns of artificially collapsed blastocysts, live birth rate did not differ between groups. However, this result should be interpreted with caution due to the small sample size and high risk of bias.

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Introduction

In mammals, preparation for the formation of the blastocoel begins in the compact morula stage embryo. In the polarized outer layer of cells, which are closely connected by tight and adherent junctions, the migration of organelles towards the inner membrane, apical localization of Na^+ channels and basolateral distribution of Na^+/K^+-ATPase provide a trans-trophectoderm Na+ gradient. This results in the osmotic movement of water into the nascent blastocoel (Watson, 1992). A growing blastocoel separates the inner cell mass (ICM) from the trophectoderm layer.

When human blastocysts are cultured *in vitro*, the blastocoel is sometimes observed to shrink or completely empty and then refill with fluid. Such partial or total blastocoel contractions have also been observed in blastocysts of other mammalian species, developed *in vivo* or *in vitro* (Cole, 1967; Kane and Bavister, 1988; Lewis and Gregory, 1929; Lindner and Wright, 1978; Massip and Mulnard, 1980).

The blastocoel can collapse spontaneously, but sometimes it can also be artificially collapsed during assisted reproductive technology procedures. Expanded human blastocysts will contract in response to any major physicochemical change in their surroundings, such as temperature drop, changes of culture media or during aspiration into a pipette or catheter. The biopsy of trophectoderm cells for preimplantation genetic diagnosis or screening usually causes the blastocyst to collapse. Furthermore, cryoprotectants used for slow freezing or vitrification of blastocysts also cause changes in blastocoel volume (Cohen et al., 1985; Fehilly et al., 1985). Blastocoel shrinkage upon cryoprotectant exposure can be quite pronounced as the cavity reaches a minimum volume in a very short period of time, and the previously expanded blastocyst assumes the appearance of a deflated ball. However, the intensity of shrinkage in cryopreservation phases before vitrification varies considerably. This led to the development of techniques for mechanical emptying of fluid from the blastocoel via micro-needle puncture (Vanderzwalmen et al., 2002), a laser pulse between two trophectoderm cells (Mukaida et al., 2006), blastocoel fluid aspiration (Chen et al., 2005) or pipetting of blastocysts with narrow pipettes (Hiraoka et al., 2004). The so-called artificial shrinkage or artificial collapsing of the blastocyst can be achieved by all these methods. Several authors have reported that artificial shrinkage before vitrification of blastocysts improves survival rate and increases the clinical success of blastocyst cryopreservation programmes (Darwish and Magdi, 2016; Hiraoka et al., 2004; Iwayama et al., 2011; Mukaida et al., 2006; Son et al., 2003; Vanderzwalmen et al., 2002). However, most of these reports were retrospective and were not optimally designed studies. Today, there is considered to be a lack of evidence as to whether or not blastocysts benefit from artificial shrinkage before vitrification. A trend towards better clinical results has been observed in two prospective randomized clinical trials (Gala et al., 2014; Van Landuyt et al., 2015). These studies did not show a significant effect on implantation and pregnancy, but did demonstrate improved survival after artificial blastocoel collapse. In addition, the Alpha consensus meeting on cryopreservation did not issue any recommendations regarding artificial blastocoel collapse (Alpha Scientists in Reproductive Medicine, 2012).

The aim of our study was to follow the behaviour of both artificially collapsed and non-treated vitrified blastocysts after warming and before embryo transfer, using time-lapse microscopy. Special attention was paid to the analysis of the blastocoel re-expansion speed and to patterns of blastocoel growth *in vitro*.

Materials and methods

Patients, ovarian stimulation, IVF and embryo/blastocyst culture

The study included IVF/intracytoplasmic sperm injection (ICSI) patients whose surplus embryos were vitrified at the blastocyst stage. During the fresh cycle, women were treated with either a long gonadotrophin-releasing hormone (GnRH) agonist (triptorelin, Diphereline; Ipsen, France) or a GnRH antagonist (cetrorelix, Cetrotide; Merck Serono, Switzerland) protocol. Oocyte retrieval was performed 35 h after human chorionic gonadotrophin (HCG) administration. The oocytes were inseminated either by IVF or by ICSI and embryos were cultured to the blastocyst stage in BlastAssist Medium (Origio, Jyllinge, Denmark) under mineral oil in an incubator with 6% CO_2 and 5% O_2, balanced with nitrogen.

Blastocyst scoring, blastocoel shrinkage and vitrification

Artificial collapsing of expanded blastocysts prior to vitrification is recommended by the manufacturer of the vitrification solution used during this study. Because this method is not yet evidence based, we have routinely practised the artificial collapse of one-half of all surplus blastocysts since 2012. In patients having at least two surplus Day 5 or Day 6 expanded blastocysts of the same grade according to our grading system (Kovacic et al., 2004), half of the blastocysts were artificially collapsed before vitrification using a laser pulse (Saturn 3, Research Instruments, UK) at the point of contact between two trophectoderm cells. The other half of the blastocysts were left intact. To prevent allocation bias, one embryologist scored blastocysts and arranged them in groups based on score. Another embryologist performed collapsing in one-half of preselected blastocysts from each scoring group.

The blastocysts were assessed as suitable for cryopreservation if they had been expanded and at least a few ICM cells were detected. Criteria for selection of blastocysts for vitrification were not strict, and so blastocysts of suboptimal quality were also vitrified. Blastocysts were vitrified individually using manufacturer protocol (Irvine Scientific, Wicklow, Ireland). In the first phase, performed at room temperature, each blastocyst was equilibrated for 10 min in an equilibration solution containing 7.5% dimethyl sulphoxide (DMSO), 7.5% ethylene glycol, 20% dextran serum supplement (DSS) and 35 µg/ml of gentamicin sulphate. In the second phase, each blastocyst was exposed to a vitrification solution containing 15% DMSO, 15% ethylene glycol, 20% DSS, 0.5M sucrose and 35 µg/ml of gentamicin sulphate for no more than 60 s. The blastocyst was then pipetted onto the curved spatula of a High Security Vitrification (HSV) device (Cryo Bio System, L'Aigle, France). The spatula was inserted into the straw, heat-sealed (closed vitrification protocol) and immersed in liquid nitrogen.

Study design, blastocyst warming and cinematographic measurements

The study was approved by the National Medical Ethics Committee on 19 April 2016 (No. 0120–204/2016–2) and conducted during 2016 at the University Medical Centre Maribor. Quasi-randomization was performed before warming, when collapsed or non-treated blastocysts were alternately selected according to even or odd dates: every

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