Evaluation of the effect of artificial collapse on the viability of vitrified bovine blastocysts

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SUMMARY

The present study aimed to evaluate the viability of the embryos following freezing and warming by vitrification after artificial collapse in bovine embryos produced *in vitro*. *In vitro* maturation, fertilization, and culture procedures were performed using oocytes obtained from ovaries collected from slaughterhouses. Embryonic development was evaluated and recorded. In total, 289 blastocysts were obtained after *in vitro* production, and 61.94% (179/289) of the obtained blastocysts were graded as Code I (excellent or good) quality. Only Code I embryos were used in the study and 60 of these blastocysts were artificially collapsed (Group 1) and 60 of them used as control (Group 2). Blastocoelic fluid of the blastocysts from group 1 was aspirated by entering through trophoblast cells using microinjection pipettes with a micromanipulator system. Thereafter, blastocysts from both groups were vitrified and warmed with ethylene glycol and glycerol-based protocols and embryonic development was monitored for 24 hours. The post-warm rate of re-expanded blastocyst was 96.66% (58/60) and 91.66% (55/60) in Group 1 and 2, respectively ($P > 0.05$). The viability rates at 24 hours were 91.66% (55/60) and 78.33% (47/60) ($P > 0.05$), and hatching rates were 65% (39/60) and 11.66% (7/60) ($P < 0.05$) in Group 1 and 2, respectively. Consequently, it was found that *in vitro* produced blastocysts can be vitrified after artificial collapse and embryo development and viability rates following warming are quite high.

KEY WORDS

Artificial collapse, blastocyst, bovine; embryo viability, vitrification.

INTRODUCTION

Embryo transfer is an assisted reproductive technology that involves the transfer of embryos obtained from high-yield donors to recipients[1,2]. Embryo production can be performed in both *in vivo* and *in vitro* conditions[3]. According to the International Embryo Technology Society (IETS), it has been reported that the total annual number of embryos produced *in vivo* and *in vitro* is 1.5 million, with greater *in vitro* embryo production compared with *in vivo* production[4].

Cryopreservation of embryos is an important step after both *in vivo* and *in vitro* production of an embryo[2,5]. This technique can be readily used if there is no favorable condition for embryo transfer, the time of transfer is not proper for recipients, and a large number of embryos have been obtained and transport or storage of the embryos is required. Moreover, cryopreservation allows the conservation of genetic resources of endangered animals and the establishment of a gene bank[6,7]. The obtained embryos can be frozen using controlled freezing (slow freezing) methods or vitrification. Vitrification is the most preferred cryopreservation method worldwide because it is cheap and fast and easily applicable[8,9]. Compared with slow freezing, vitrification is used more often because it results in greater enhancement of post-warm re-expansion and hatching rates of embryos produced *in vitro*[6,10].

The embryo reached the blastocyst stage is the optimal time for cryopreservation because of its high cell count and differentiation[11]. Similar to slow freezing, the initial condition in vitrification is cellular dehydration. Water is excreted through osmosis from the water channels called the aquaporin of the embryo exposed to hypertonic cryoprotectant substances. The embryo rapidly shrinks owing to the outflow of water in the embryo and the entry of cryoprotectants into the cell. The second osmosis occurs when the embryo begins to be exposed to subzero degrees after achieving an intercellular fluid balance. However, because of the fluid-filled cavity called blastocoel, the blastocyst is sensitive to freezing. It has been emphasized that the survival ability of the embryo decreases after vitrification owing to the increased volume of the blastocoel. As the blastocoel volume increases, the influx of cryoprotectants into the blastocyst becomes insufficient, leading to an increased risk of ice crystal formation. The liquid remaining in the blastocoel reduces the survival ability of the embryo during thawing[6,11]. Owing to these reasons, when the embryos obtained from humans

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need to be frozen, artificial collapse (AC) is routinely performed as first mentioned by Vanderzwalmen et al. (2002)[12] and it has been reported that subsequent vitrification procedures reduced DNA damage in the embryo and that post-thaw re-expansion and ability of survival were more successful[12-17]. In this point of view the applicability of this method in *in vitro* produced bovine embryos was tried to be determined. Also, the present study evaluated the effect of artificial collapse prior to vitrification on the viability, development, and hatching process of the *in vitro* produced embryos following warming.

MATERIAL AND METHODS

The study was approved by the Local Ethics Committee (Ankara University Local Ethics Committee for Animal Experiments, Approval Number: 2016-5-73).

Collection of bovine ovaries and oocyte retrieval

Ovaries of Holstein breed cows were collected from a slaughterhouse in Ankara, Turkey and transferred to the laboratory in 0.9% isotonic NaCl solution (Deva, Turkey) containing 50 µg/ml gentamicin (Sigma-Aldrich, USA) at 20°C for a maximum of 4 hours. The ovaries were washed at least twice with 0.9% NaCl solution to remove the surrounding tissues, blood, and transport medium. Washed ovaries were left in isotonic solution until oocyte aspiration. The follicle fluid containing the cumulus-oocyte complexes (COCs) was aspirated from the 2-8-mm diameter peripheral follicles found in the ovaries using an 18G needle with a pressure adjustable (180-200 mmHg) aspiration pump. The collected follicle fluids were taken into conical centrifuge tubes. These tubes were kept for 5-10 minutes at the end of the aspiration procedure to allow the oocytes to settle to the bottom. COCs accumulated at the bottom of the tubes were washed two times in medium containing 1% calf serum (CS, Sigma-Aldrich, USA) + Ringer's solution (Polifarma, Turkey) to separate them from other cells in the tubes. After washing was completed, the medium containing COCs was transferred into 90-mm diameter Petri dishes and examined under a stereomicroscope. The obtained COCs were evaluated as previously described[18].

In vitro maturation

COCs detected under the microscope were collected and placed into 60-mm Petri dishes containing medium (1% CS + Ringer's solution). Collected COCs were transferred into 35-mm Petri dishes containing 25 mM HEPES-buffered M-199 (Sigma-Aldrich, USA) + 5% CS + 0.02 mg FSH (Folltropin-V, Vetoquinol, Canada) + antibiotic (maturation medium) after washing at least five times at different points of the 60 mm Petri dishes. Then, COCs were taken into maturation drops (5 µl for each oocyte) and each drop contained 20 oocytes. Oocytes were matured in an incubator at 38.5°C with 5% CO_2 for 22-24 hours at maximum humidity.

Table 1 - Re-expansion, viability and hatched rates in blastocysts following vitrified/warmed.

	Number of Blastocysts (n)	Re-expanded blastocysts (%)	Viable blastocysts (%)	Hatched blastocysts (%)
Group 1 (AC)	60	58 (96.6)	55 (91.6)	39 (65)
Group 2 (Control)	60	55 (91.7)	47 (78.3)	7 (11.6)
P		>0.05	>0.05	<0.05

In vitro fertilization

Frozen semen of an only one Holstein bull was used for sperm treatment in the study. Before *in vitro* fertilization, Percoll (Sigma-Aldrich, USA) gradient technique was used to select motile spermatozoa[19]. The final sperm concentration was determined as 3×10^6/ml. Afterward, the sperm suspension prepared for *in vitro* fertilization was taken into 35-mm Petri dishes in drops and covered with paraffin liquid. The COCs extracted from the maturation medium were washed twice in Brackett and Oliphant's solution containing 10 mg/ml BSA (Sigma-Aldrich, USA), then taken into drops contained fertilization medium (Tyrode lactate solution supplemented with BSA, Sodium pyruvate, penicillin, streptomycin) and cultured in an incubator at 38.5°C with 5% CO_2 at maximum humidity for 18-20 hours (Day 0).

In vitro culture

After fertilization, presumptive zygotes were washed in embryo culture medium. Denudation was performed for the removal of the cumulus cells and sperm. Presumptive zygotes that were completely peeled from the cumulus cells were then transferred into the culture medium. CR1aa + 5% CS + 0.25 mg/ml linoleic acid albumin (Sigma-Aldrich, USA) culture medium were used for embryo culture[20]. Presumptive zygotes taken into embryo culture medium drops were cultured in an incubator at 38.5°C with 5% CO_2 at maximum humidity for 7 days.

Evaluation of obtained embryos

Day-7 embryos after *in vitro* culture were evaluated according to the IETS criteria[21]. Accordingly, the codes for embryo quality were graded as follows: Code I (excellent or good) represents symmetrical and spherical embryo mass, uniform blastomeres in size, color, and density, and ≥85% intact cellular material; Code II (fair) represents ≥50% intact cellular material and vital embryonic mass, moderate irregularity of the blastomeres in size, color, and density, and ≤25% fragmentation; Code III (poor) represents marked irregularity of the blastomeres in size, color, and density and ≥25% intact cellular material and vital embryonic mass; Code IV (death or degenerated) represents degenerated embryos and unfertilized oocytes. Code I quality blastocysts were randomly selected; 60 of these embryos were used for AC (Group 1), and 60 were used only for vitrification without AC (Group 2, control).

Artificial Collapse

On day 7/8 of embryo development in the Group 1, blastocoelic fluid was aspirated with a micromanipulator (Olympus, IX73, Japan) and microinjection pipette (Eppendorf TransferMan 4R, Germany) in embryo culture medium[22]. During this procedure, all of the fluid was aspirated by a microinjection pipette inserted between the trophoblast cells without interference with the inner cell mass until the blastocoel was completely collapsed. After that, the blastocysts immediately transferred into vitrification solution. No manipulation was applied to the embryos of the control group.

Vitrification of the embryos

Embryos in both groups were cryopreserved by vitrification. First, embryos were transferred into the vitrification solution (VS)-1 (10% Glycerol + 0.1 M Sucrose + 1% BSA) and kept for 5 minutes. Afterward, embryos were taken into the VS-2 (10% Glycerol + 10% Ethylene Glycol + 0.2 M Sucrose + 2% BSA) and kept in this solution for 5 minutes; finally, following the transfer to the VS-3 (10% Glycerol + 10% Ethylene Glycol + 0.3 M Sucrose + 3% BSA), embryos were pulled to the 0.25 ml straws (one straw for one embryo) within 1 minute and the open part of the straw was closed by a plug. Later, the straw was placed into liquid nitrogen and vitrification was completed.

Warming of the embryos

For devitrification, the straws protected in the liquid nitrogen were warmed in two phases, for 10 seconds in the air and 20 seconds in a 30°C water bath. Subsequently, to purify the toxic level of sucrose in the embryo, they were initially transferred into a Petri dish containing mD-PBS (Sigma-Aldrich, USA) 0.5 M sucrose CS and washed for 5 minutes. Then, washing was done with lesser concentrations of mD-PBS + 0.25 M sucrose + 20% CS solution for 5 minutes. In the final stage, equilibration was performed in mD-PBS + 20% CS solution for 5 minutes. After devitrification, embryos were placed into 25 µl drops of M-199 + 20% FCS + 0.1 mM β-mercaptoethanol (Sigma-Aldrich, USA) for culture and incubated at 38.5°C with 5% CO_2 for 24 hours. During the 24-hour incubation period, the development of embryos was observed under a stereomicroscope. During incubation, the re-expansion of embryos and achievement of the advanced embryonic developmental stage (expanded, hatched embryo) were used to determine the viability of these embryos. In addition, embryos were followed up in terms of hatching during the first 24 hours.

Statistical analysis

For discrete and continuous variables, descriptive statistics were given. In addition, the homogeneity of the variances, which is one of the prerequisites of parametric tests, was checked through Levene's test. The assumption of normality was tested via the Shapiro-Wilk test. To compare the differences between two groups, Student's t test was used when the parametric test prerequisites were fulfilled, and the Mann Whitney-U test was used when such prerequisites were not fulfilled. The data were evaluated via SPPS 25 (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). $P<0.05$ and $P<0.01$ were taken as significance levels.

RESULTS

In total, 289 blastocysts were obtained in the study. 179 of these embryos were evaluated as Code 1 (61.94%), 51 of them as Code 2 (17.65%) and 39 of them as Code 3 (13.49%). Only Code I quality embryos were used in the study and 60 of these embryos were included in group 1 and 60 in group 2.

After the vitrified embryos were warmed according to the procedure, they were transferred back to the culture medium and the developmental processes were evaluated for 24 hours. The rates of re-expansion, viability, and hatched blastocysts at the end of 24 hours are given in Table 1. The re-expansion of embryos and achievement of the advanced embryonic developmental stage during incubation indicated the viability of these embryos. No statistical difference was found between re-expanded and viable blastocyst rates in the AC and control groups. However, the rate of hatched blastocysts was higher in the AC group compared to the control group ($P<0.05$).

DISCUSSION

Intracellular ice formation or high concentration compounds used during cryopreservation affects the transcription of developmentally important genes and the viability of the cells, thus reducing the survival ability of the embryo and the rate of conception[9,10]. One of the methods that can be used to eliminate these problems is to reduce the blastocoelic fluid before vitrification[12]. Artificial collapse of the blastocyst before vitrification is a new approach to improve the viability of the blastocyst after warming. This technique helps to reduce the damage caused by ice crystal formation by reducing the amount of fluid in the blastocoel[15]. Several studies have reported higher rates of re-expansion, viability, and hatching of the embryos frozen or thawed after the blastocoelic fluid was reduced[12,14,15,23]. This study evaluated the effect of this method, which was previously used in humans, on development of bovine embryos produced *in vitro*.

According to the obtained findings, the re-expansion rates after vitrified/warmed were 96.7% and 91.6% in Group 1 and 2, respectively ($P > 0.05$). The reason for the lack of difference between the re-expansion rates is thought to be due to the recovery of blastocoelic fluid following devitrification in both groups. However, it is thought that there were problems in the steps after re-expansion in the control group. Returning to the pre-freezing phase is very critical for the embryos to continue their development after freezing/thawing[24]. There is a close relationship between the success of vitrification and the expansion of the blastocoel because the large blastocoel before freezing causes insufficient cryoprotectant permeation and dehydration[12]. Kovacic et al. (2018)[25] have reported that the blastocyst volume inside the zona pellucida was significantly lower in the group with artificial collapse following warming than in the control group. However, the large volume of blastocysts in the control group is thought to be owing to partially persistent fluid accumulation before vitrification. However, this persistent fluid may have a toxic effect on the embryo after warming[25]. Desai et al. (2008)[15] have reported greater cell damage in blastocysts without artificial collapse than in collapsed blastocysts. This may be because of insufficient dehydration of the blastocoel and a slower rate of recovery/re-expansion of non-collapsed blastocyst[15].

In the present study, survival rates after vitrified/warmed were 91.6% and 78.3% in groups 1 and 2, respectively ($P > 0.05$). Although there was no statistical difference between the survival rates of the embryos in both groups, the survival rate in the AC group was higher than in the control group. The reason for this low blastocyst viability rate in the control group is thought to be due to the ice crystals formed during vitrification. Because blastocoelic fluid influence the permeation of cryoprotectant during vitrification, and this cause intracellular ice crystal formation. Also, Ha et al. (2010)[26] and Min et al. (2013)[27] have found that artificial collapse treatment increases the survival rate in cattle. In previous studies by Desai et al. (2008)[15] and Levi-Setti et al. (2016)[28] in humans and Kazemi et al. (2016)[29] and Pooyanfar et al. (2018)[30] in mice, it was re-

ported that the survival rate did not improve by artificial collapse of blastocysts. However, in these studies, artificial collapse was performed with the microneedle technique. In contrast, studies have reported that artificial collapse with laser pulse leads to an increase in the survival rate[31,32]. Moreover, Van Landuyt et al. (2015)[23], Darwish and Magdi (2016)[33], and Kovacic et al. (2018)[25] have noted increased survival rates after artificial collapse using a laser pulse. This may be owing to the lower probability of damage to the trophectoderm cells of the embryo with a laser pulse[33]. However, Wang et al. (2017)[32] have reported that artificial collapse of blastocysts using a microneedle and laser pulse did not affect the survival rates of the embryos.

In the present study, the rates of hatched embryos were quite high in Group 1 compared with Group 2 (65% vs. 11.6%, respectively; $P < 0.05$). The higher rate of hatched blastocyst in the AC group is thought to be due to the higher survival rate in this group and the lower rate of damaged cells. Because embryos with a lower rate of damaged cells continue to divide and reach the hatched stage. Thus, vital cells and contractions are essential for the hatching of the embryo. However, damaged cells observed in the embryos subjected to standard vitrification may preclude the hatching process. The lesser number of damaged and apoptotic cells in embryos subjected to artificial collapse can facilitate the hatching process[25,27,30]. Min et al. (2014)[34] found that the implementation of forced collapse prior to vitrification increased the hatching rate after warming in their study on cattle. Ha et al. (2010)[26] reported that the hatching rates of the embryos at the 12th hour after warming in the group with artificial collapse prior to vitrification and in the control group were 51.7% and 0%, respectively. In previous studies by Cao et al. (2014)[31] and Van Landuyt et al. (2015)[23] in humans and Kazemi et al. (2016)[29] in mice, it was reported that higher hatching rates in embryos were observed with artificial collapse. Therefore, it has been established that the implementation of artificial collapse before vitrification is effective in increasing the hatching rates after warming.

CONCLUSIONS

The reduction of blastocoelic fluid by a micromanipulator in *in vitro* produced bovine embryos has no statistically significant effect on the re-expansion and survival of post-warm embryos; however, improved hatching rate was observed in the group with artificial collapse. Therefore, the implementation of artificial collapse before freezing of embryos produced *in vitro* by vitrification may positively affect post-warm embryo development and hatching. Nevertheless, future studies involving cattle are needed to evaluate the effect of this procedure on the conception rate after the transfer.

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