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Specifics of vitrification of *in vitro*-produced cattle embryos at various development stages

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Producing embryos *in vitro* is an important technology used to improve the genetic potential of cattle and perfect the programs of their breeding. Regardless of the way they are produced, all embryos that had not been used for transplantation to recipients must be conserved. Because of significantly increased interest in the problem of cryoconservation of embryos, both coming from scientists and businesses, there are emerging new commercial environments that allow the facilitation of cryoconservation and the increase in the embryo survival. Oocyte-cumulus complexes obtained from the ovaries of slaughtered clinically healthy cows matured in 22–24 h in *in vitro* conditions. The oocytes were co-cultured with spermatozooids in Fertilization medium, and the obtained zygotes were cultured in Culture medium with Sodium-Pyruvate for 4 or 7 days up to the stage of morula or blastocyst, respectively. For the vitrification of cow embryos, we used a commercial kit for the vitrification of human embryos, having compared the duration of equilibration. According to the results of the studies, we observed high efficiency of cryoconservation of cow embryos using the commercial kit for vitrification of human embryos. The results revealed the significant effect of equilibration on survival and further development of embryos. In addition, we described the dependence of development stage of cattle embryo on the duration of the contact of embryo with equilibration solution. Therefore, optimal time of contact of cattle embryos at the morula stage with equilibration solution was 12 minutes. On the 24th h after thawing, 46.7 ± 3.3% of the embryos were observed to undergo blastulation, and on 48th h, this parameter increased to 96.7 ± 3.3%, which corresponded to the parameters in the group of embryos that had not been subjected to cryoconservation. In the conditions of further cultivation, the percentage of blastocysts that hatched in the experimental group was no different from that of the control. At the same time, the highest efficiency of vitrification of blastocysts of cows was seen after the contact with the equilibration solution for 15 min, since the percentage of hatched blastocysts was the same as in the control group. Therefore, using the commercial kit for vitrification of human embryos is beneficial, for it promotes the parameters of cow embryos after vitrification/thawing that are similar to such of intact embryos (without freezing). The data we analyzed and presented in the paper could help to increase the efficiency of cryoconservation of cattle embryos for both scientific and commercial purposes.

Keywords: equilibration; protocol of cryoconservation; biotechnology of reproduction of ruminants; morula; blastocyst.

Introduction

In 2020, the world's population reached 7.7 B people, and is expected to increase up to 8.9 B in 2035 and 9.2 B in 2050 (Raney et al., 2009). This, in turn, would require significant increase in the provision of food supplies. Improvement of agricultural methods becomes increasingly important for food provision in the world, for animal protein is first of all necessary for the correct cognitive development of children. As a result of increase in human population and demand for limited resources, the cost of energy carriers and concentrates would increase. The elements most economically impactful on profitability of livestock breeding sphere are fodders, which account for over 40% of the production costs. The main contribution to the stability of the sphere is efficiency in fodder consumption, since it affects economic and ecological parameters (Siberski-Cooper & Koltes, 2021).

The global changes in the environmental conditions due to climate change (Petrescu-Mag et al., 2022) makes it necessary for us to pay more attention to new approaches to managing herds, especially aspects of optimizing the efficiency of using the generative resources. Cattle-holding industries would have to search for new methods of reducing emissions of

greenhouse gasses during the production cycle by increasing the effectiveness of the production and decreasing wastes, or seek for new technologies to minimize contamination. In this context, there is a need of new indicators that could have been used in management and breeding of herds (Egger-Danner et al., 2015). Therefore, in particular, increases in milk production per dairy cow have led to decrease in the need of energy per unit of produced milk, and therefore lessened CH₄ emissions per unit of milk (Capper et al., 2009; Gerber et al., 2013). However, further progress is possible only by introducing new developments, including those that improve genetic traits of herd (Lövendahl et al., 2018). Inclusion of functional traits in the program of genetic improvement is important for long-term development of dairy populations. Genetic achievement is cumulative, so insignificant improvements build up over time and thus would provide stable economy to all farmers, especially those using selection indices to combine data from multiple various traits (Egger-Danner et al., 2015). Nonetheless, changes in certain traits resulting from selection breeding are occurring at relatively low rate (0.5–3.0% per year) (Jennifer et al., 2013). At the same time, reproductive biotechnology that would induce changes could be implemented at fast rates by intensifying the selection, and not only reproduction rates. Therefore, the technology of

genome selection performed by selection breeding companies increases the demand of embryos obtained from genetically desired animals, which shortens the intervals of generation and increase of genome increment (Aardema et al., 2022).

Currently, embryo transplantation is an important technology used for genetic improvement and cattle breeding programs (Dochi, 2019). There are two methods of obtaining cattle embryos: *in vivo* and *in vitro*. Until 2016, embryos have been predominantly produced *in vivo* (Viana, 2020), though scientists noted that producing oocytes by ovum pick up (OPU) makes it possible to obtain larger amounts of *in vitro*-derived embryos per animal compared with multiple ovulation and embryo transfer (MOET), which is due to variability in donors, which hinders the prognoses of the number of *in vivo* embryos (Boland et al., 1991; Hasler et al., 2003; Ferré et al., 2020). Therefore, there has been a rapid increase in the number of *in vitro*-produced cattle embryos around the globe, up to 1 M in 2019 against the background of decrease in the number of embryos produced *in vivo* (378,769 in 2019) (Viana, 2020). In order to manage such an amount of embryos, cryoconservation is used, which is a perfect tool logistics-wise, since transplantologists can optimally choose the time of transplanting the embryo with reproductive cycle to the recipient and transport it large distances. Unfortunately, the cryosurvival level of *in vitro*-produced embryo is lower compared with embryos obtained in *in vivo* conditions (Moore & Hasler, 2017; Ferré et al., 2020; Viana, 2020).

Regardless of production method, all embryos that had not been transplanted to recipients are cryoconserved. The technology of embryo cryoconservation is one of the most important achievements in transplantation of cattle embryos (Hasler, 2014). The study of technology of freezing cattle embryos began in 1973, when Wilmut and Rowsin obtained calves from frozen-defrozed embryos for the first time (Wilmut & Rowsin, 1973). Currently, there are two protocols: slow freezing and vitrification (Valente et al., 2022). Slow freezing was developed in the 1970s (Hasler, 2014), whereas vitrification was for the first time performed in 1985 (Rall & Fahy, 1985). Slow freezing using low concentration of permeating cryoprotectant is broadly used to store cattle embryos (Hasler, 2014; Janati Idrissi et al., 2021). Despite the reliability of protocols of cryoconservation of *in vivo*-derived embryos, they are less effective in the work with *in vitro*-produced embryos (Dobrinisky, 2002; Hasler, 2014). The vitrification methods are currently seen to be undergoing improvements (Hasler, 2014; Rienzi et al., 2017; Arshad et al., 2021), and furthermore, this method of cryoconservation has several advantages specifically for *in vitro*-derived embryos. Secondly, rates of re-expansion, hatching of embryos produced by extracorporeal fertilization and the survival rate of vitrification-frozen embryos were higher than those of embryos frozen using the method of slow freezing (Nedambale et al., 2004; Mucci et al., 2006; Najafzadeh et al., 2021). Also, this technology reduces the cryodamage to oocytes and membranes of cattle (Vajta et al., 1998; Inaba et al., 2016; Janati Idrissi et al., 2021) and decreases the sensitivity of embryos to freezing (Mahmoudzadeh et al., 1994). Another peculiarity of vitrification is that it prevents the formation of ice crystals during the freezing process by increasing the rates of freezing and warming (Vajta et al., 1998; Estudillo et al., 2021). Thirdly, vitrification is cheaper than stepwise methods (Martinez-Rodero et al., 2021). Fourthly, it is very simple to perform, does not require complicated equipment, and the technology is easy to learn over a short practical course (Campos-Chillon et al., 2006). Moreover, frequency of pregnancy of recipient cows in the conditions of using vitrified *in vitro*-derived embryos is appropriate for commercial purposes (Liebermann et al., 2006; Arshad et al., 2021).

Vitrification of embryos at various stages of the development is being debated (Juanpanich et al., 2018; Do, & Taylor-Robinson, 2020). Using cryoconservation of blastocysts, embryos can be transferred right after freezing, avoiding the re-cultivation of embryos in *in vitro* conditions (Aono et al., 2013). Asgari et al. (2012) found that 5–8-cellular embryos stopped developing, whereas 8–16-cellular morules became blastocysts, which is explained by the influence of the development kinetics. Shirazi et al. (2009) demonstrated that early blastocysts have higher cryotolerance than the expanded ones. However, Sommerfeld & Niemann (1999) indicate that expanded blastocysts survive after vitrification better than non-expanded – 42% and 12%, respectively. On the other hand, cryoconservation of embryos at early stage slows the *in vitro* production of embryos,

since it is important that those embryos continue to be *in vitro* cultivated after thawing, developing to the blastocyst phase, when they can be transplanted to recipients (Do & Taylor-Robinson, 2020).

Despite the fact that many aspects of cryoconservation of cattle embryos have been described in the literature, the recent interest in this problem coming from both scientists and entrepreneurs has led to emergence of new commercial solutions that facilitate the cryoconservation procedure and the increase the survival rate of the embryos.

Because of absence of scientific data on the effects of equilibration period of cattle embryos on their survival and further development after using commercial kit Cryotech Vitrification Kit for vitrifying human embryos (Cryotech, Japan), we conducted vitrification of cattle embryos at various development stages so as to determine the optimal equilibration time.

Materials and methods

Production and maturation of oocyte-cumulus complexes. The ovaries from clinically healthy cows were selected in the slaughter house and transported in a thermos at the temperature of 30–33 °C no more than 3 h after the extraction. In the laboratory, the ovaries were 4 times rinsed in sterile Dulbecco's Phosphate Buffered Saline (Sigma, USA) with addition of 0.075 mg/cm³ of kanamycin sulfate (Sigma, USA) (the temperature of the solution was 37–38 °C). Removal of oocyte-cumulus complexes from the antral follicles (2–8 mm in size) of the ovaries of cows was performed in a laminar box by cutting the follicles with a safe blade in the oocyte collecting solution comprising 5 cm³ of TL HEPES (Minitube, Germany) with addition of 30 mg of bull serum albumin (Sigma, USA). Removal of oocyte-cumulus complexes, their selection and maturation, insemination and further cultivation were also carried out in the conditions of sterile box. After evaluation under SZ51 stereomicroscope (Olympus, Japan), we selected 120–130 μm of oocyte-cumulus complexes, with entire dense cumulus, undamaged transparent membrane and homogenous non-vacuolated ooplasm of regular round shape, without noticeable morphological signs of atresia. The extracted oocyte-cumulus complexes were 6 times rinsed in the solution for collecting oocytes. The oocyte-cumulus complexes were selected and rinsed on a heating table at the temperature of 37 °C. The oocytes had been maturing in *in vitro* conditions for 22–24 h in 4-well dishes (Oosafe, USA). Into each well, we added 300 mm³ of the solution comprising 4.5 cm³ of the initial TCM 199 (Minitube, Germany) solution for maturation, 0.5 cm³ of estrous cow serum 125IU of folliclestimulating hormone and 1.25IU of luteotropic hormone (50 mm³ of Pluset (Laboratories Calier S.A., Spain) and 0.125 IU of folliclestimulating hormone (FSG-super (Agrobiomed, Russian Federation) and 50 mm³ of antimycotic antibiotic (Sigma, USA), and then it was covered by mineral oil (Origio, Denmark), added 25 oocyte-cumulus complexes and cultivated in a CO₂-incubator at the temperature of 38.5 °C and 6% CO₂ and 5% O₂.

Preparing sperm to insemination. Preparation of bull's sperm to insemination was performed using density gradient Origio Gradient Series (Origio, Denmark) and solution for capacitation of spermatozooids (Minitube, Germany). Constituents of gradient were heated up to the room temperature (20–25 °C), and all other reagents were equilibrated in a CO₂-incubator at the temperature of 38.5–39.0 °C and 5% CO₂ for no less than 2 h. The gradient was prepared by carefully layering 1 cm³ of Origio Gradient 80 on 1 cm³ of Origio Gradient 40, and then sperm that had been defrozed on a water bath was carefully introduced. The formed system was centrifugated at centrifugal force of 300 g for 20 min. The supernatant was removed and the sediment was transferred using a sterile nozzle to a test tube with 2 cm³ of the solution for preparation and capacitation of spermatozooids, consisting 5 cm³ of basic solution for capacitation (Minitube, Germany), 30 mg of bovine serum albumin (Sigma, USA), 0.55 mg of sodium pyruvate (Sigma, USA) and 50 mm³ of antimycotic antibiotic (Sigma, USA), and then was centrifugated at centrifugal force of 300 g for 5 min, and most of the supernatant was removed. The procedure was repeated twice. After rinsing, the sediment was transferred to the bed of the test tube with 1 cm³ of new portion of the solution for preparation and capacitation of spermatozooids. Obtaining mobile fraction of spermatozooids was performed using the swim-up method described by Parrish et al.

(1986). One-hour incubation was enough for mobile spermatozooids to elevate to the upper layers of the solution, while dead and pathological ones remained at the bottom of the test tube. Mobile spermatozooids were capacitated in solution for preparation and capacitation in the conditions of 4 h influence of heparine (Sigma, USA) in the concentration of $20 \mu\text{g}/\text{cm}^3$ in the CO_2 -incubator at the temperature of 38.5°C with 6% CO_2 and 5% O_2 . After capacitation, the spermatozooids were centrifugated at centrifugal force of 200 g for 5 min. We removed the supernatant and added 1 cm^3 of the solution for insemination and determined the concentration of spermatozooids in Goryaev's chamber hemocytometer.

Co-cultivation of oocytes and spermatozooids. After maturation, the oocytes were co-cultured with spermatozooids. Insemination of oocytes was carried out in 4-well dishes (Oosafe, USA) in 300 mm^3 of solution composed of 5 cm^3 solution for fertilization (Minitube, Germany), 30 mg of bovine serum albumine, $0.11 \mu\text{g}$ of sodium pyruvate, 0.2 mg of heparin and 50 mm^3 of antimycotic antibiotic (Sigma, USA). It was coated by mineral oil (Origio, Denmark) for 18 h after the addition of capacitated spermatozooids (in calculation of 1×10^6 mobile spermatozooids/ cm^3). The number of oocytes in well varied 5 to 10.

Cultivation of embryos. After the co-cultivation, oocytes of cattle which had swollen expanded cumulus were freed from cumulus cells using the method of soft pipetting in 0.1% of hyaluronidase solution (Sigma, USA). Then, the oocytes were rinsed from the enzyme in 5–6 drops of TL HEPES (Minitube, Germany) and transferred to the cultivation solution, which was composed of 5 cm^3 of growth medium with pyruvate (Minitube, Germany), 0.5 cm^3 of estrous cow serum, 200 mm^3 of essential amino acids (Sigma, USA), 50 mm^3 of non-essential amino acids and 50 mm^3 of antimycotic antibiotic (Sigma, USA). Embryos were cultivated in 65 mm^3 of microdrops (3 embryos in one drop) under the layer of mineral oil (Origio, Denmark) in cultivation dishes (Oosafe, USA) in CO_2 -incubation in the temperature of 38.5°C with 6% CO_2 and 5% O_2 . Inseminated oocytes were determined according to the division of 2–8-cellular stages 48 h after the contact with spermatozooids. Embryos reached the morula stage on the 3rd day of cultivation, and blastocysts on the 6–8th days.

Vitrification. Vitrification was performed for embryos of two development stages, according to the classification proposed by Bó & Mapletoft (2013), recommended by the International Embryo Technology Society (IETS) (Rocha et al., 2016), morula – cycle day 4, stage code 3 (cellular mass comprised of no less than 16 cells, some blastomeres were hard to distinguish one from another, cellular mass of embryo accounted for most of the perivitelline space), quality code 1 (embryo had symmetric and spherical mass, which comprised separate blastomeres of the same size, colour and density, it corresponded to the expected development stage; irregularities were relatively insignificant, and at least 85% of the cellular material had undamaged vital embryonic mass; the zona pellucida was smooth and had no concavities or flat surfaces, Fig. 1) and blastocyst – cycle day 7, stage code 6 (we observed a notable differentiation of trophoblast – outer layer of cells and embryoblast – compact inner mass, blastocoel was strongly expressed, embryo occupied most of the perivitelline space), quality code 1, Fig. 2).

Quality of embryos was evaluated under inverted microscope Zeiss Axio Observer A (Carl Zeiss, Germany), photos and measurements were taken using Octax Navilase laser system (OCTAX Microscience GmbH, Germany).

In order to cryoconserve cattle embryos, we used Vitrification Kit 101 (Cryotech, Japan), which comprises equilibration solutions (ES) and vitrification solution (VS), 3-well dishes Vitri, Cryotec. Prior to the use, reagents were held for at least 1 h in the room temperature. Taking into account the data of Cryotech protocol, where maximal exposure time in the solution for equilibration was 15 min for oocytes and blastocysts and 12 min for other stages of embryonal development, we decided to compare the effects of 10, 12 and 15 min equilibration on the further development of cattle embryos.

Prior to the start of cryoconservation procedure, the container for freezing was filled with liquid nitrogen and marked Cryotec. For the procedures with embryos, we used Stripper micropipette (CooperSurgical, USA) with flexible polycarbonate nozzles Flexipet (Cook, USA), 170 or 300 μm , depending on the size of embryo. The first well of the dish was

filled with 300 μL of the equilibration solution (ES); the second and the third – 300 μL of the vitrification solution (VS). Using the pipette, we distributed the embryos on the surface of the equilibration solution. Because the objective of our study was determining the influence the equilibration time takes on the effectiveness of freezing, the morules (overall number – 120) and blastocysts (total of 120) were divided into 4 groups, with different time of embryo contacting ES – 10, 12 and 15 min, and embryos that were not subjected to vitrification (the control group). To confirm the significance of the obtained data, the experiment was repeated three times.

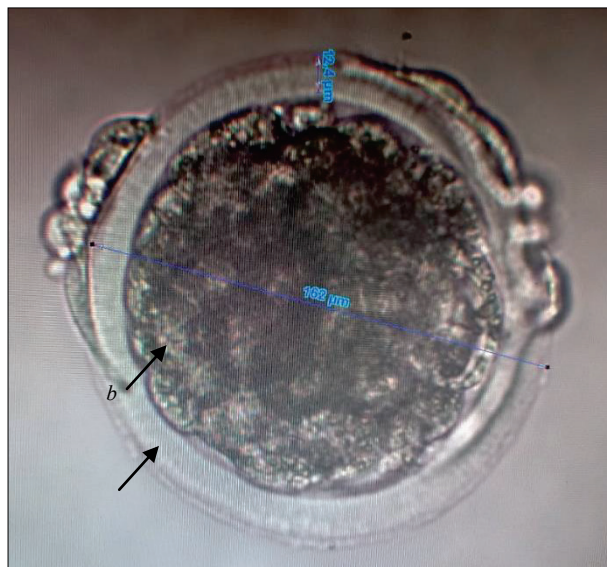


Fig. 1. Morula of cattle prior to vitrification (cycle day – 4, stage code – 3, quality code – 1): zona pellucida (a), compact cell mass (b)



Fig. 2. Blastocyst of cattle prior to vitrification (cycle day – 7, stage code – 6, quality code – 1): zona pellucida (a), blastocyst cavity (b), inner cell mass (c), trophoblast (d)

The next steps lasted over 25 s, but no longer than 90 s. After the equilibration, embryo aspiration was performed with a small amount of equilibration solution and transferred to another well (vitrification solution), descending the embryo on the middle depth of the solution. The pipette was rinsed from the solution. The embryo in this solution surfaced, and therefore it was 5–6 times suctioned with pipette and put onto the bottom of the well.

When the embryo stopped surfacing, it was transferred to the third well on the middle depth of the vitrification solution. It was rinsed with fresh vitrification solution and the mixture around the embryo was stirred, expecting its complete sedimentation on the bottom of the well. We suc-

tioned the embryo with vitrification solution on the pipette end and put it near the black marking on the Cryotec in minimum volume of liquid (less than 0.1 μ L). We removed the excessive amount of the vitrification solution by pulling the pipette end in the lower part of the large drop of the vitrification solution, moving the pipette in horizontal direction, thereby expanding the drop and carried out the aspiration of the excess of the liquid by decreasing the drop as much as we could. Under a stereomicroscope, we checked that embryo is on the Cryotec with minimal volume of vitrification solution and quickly submerged it into liquid nitrogen. Having not taking it out of nitrogen, we covered the Cryotec by a protective lid and transferred it into cryogenic storage dewar for maintenance.

Thawing. To thaw the cattle embryos, we used Warming Kit 102 (Cryotech, Japan), comprising solutions for thawing (TS), diluent (DS) and washing (WS), 4-well Warm Plate. Prior to using DS, WS, we held it for at least 1 h in the conditions of room temperature. Prior to use, TS was heated in a thermostat at 37 °C for 3 h. Into well No. 2, we introduced 300 μ L of DS, into wells No. 3 and 4 – 300 μ L of WS into each. We prepared Cryotec to thawing (we took the protective lid off using tweezers in the conditions of liquid nitrogen). We introduced TS into well No. 1 right before the thawing and quickly (in 1 s) submerged the Cryotec into well No. 1 and waited 1 min. Then, using Stripper micropipette (CooperSurgical, USA) with a flexible 170 or 300 μ m polycarbonate nozzle, regardless of the embryo size, we transferred it to well No. 2 and left it there for 3 min. After the indicated period, the embryo was transferred to well No. 3 for 5 min, then – to well No. 4 for 1 min. The final stage of thawing was transferring the embryo into cultivation solution. After thawing, we evaluated the development status of embryo every 24 h.

The obtained results were statistically analyzed in ANOVA software, the data in tables are presented as $x \pm SE$ (mean \pm standard error). To compare the difference between the parameters of the control and experimental groups, we used the Tukey test, where the differences were considered statistically significant at $P < 0.05$ for all the data.

Results

According to the results of the studies, we determined that the period of contact with equilibration solution in the vitrification conditions caused various levels of survival of the morules. Analysis of the parameters of blastulation and hatching of embryos revealed some differences in the development of embryos of the experimental groups 2 and 4, compared with the control (without freezing, Table 1).

Table 1
Effect of morules' equilibration period when using Cryotech Vitrification Kit on their survival and further development ($x \pm SE$, $n = 3$)

Period of equilibration, min	Blastocysts on the 24 th h after thawing, %	Blastocysts on the 48 th h after thawing	Blastocysts that did not hatch, %
Control (without freezing)	43.3 \pm 3.3 ^a	96.7 \pm 3.3 ^a	90.0 \pm 5.7 ^a
10	36.7 \pm 3.3 ^b	90.0 \pm 5.7 ^b	83.3 \pm 3.3 ^a
12	46.7 \pm 3.3 ^a	96.7 \pm 3.3 ^a	90.0 \pm 0.0 ^a
15	33.3 \pm 3.3 ^b	83.3 \pm 3.3 ^b	73.3 \pm 3.3 ^b

Note: letters indicate significant differences between the groups within a column ($P < 0.05$) according to the results of the Tukey test.

Equilibration period lasting 12 min appeared to be optimal for vitrification of cattle morules. We obtained 46.7 \pm 3.3% of blastocysts in the 24th h after thawing and 96.7 \pm 3.3% in the 48th h, which corresponded to the parameters of the control, equaling 43.3 \pm 3.3% and 96.7 \pm 3.3%, respectively. Hatching rate of blastocysts in this experimental group did not differ from those we observed for fresh non-vitrified embryos (90.0 \pm 0.0% against 90.0 \pm 5.7%, respectively). The obtained parameters indicate the high efficiency of vitrification of cattle morules using the Cryotech kit in the conditions of 12 min exposure to equilibration solution.

Two minute decrease in the equilibration period led to decrease in the parameters of the embryos' development. Therefore, the percentage of blastulation was 10% lower on the 24th h after thawing, 6.7% lower on the 48th h, compared with group of embryos where the equilibration lasted 12 min. The percentage of hatched blastocysts was also 6.7% lower. At the same time, in the group where the embryos had been subjected to

the action of equilibration solution for 15 min, we observed significantly lower percentage of formation of blastocysts in the 24th and 48th h after thawing – 33.3 \pm 3.3% and 83.3 \pm 3.3%, as well as hatching of blastocysts – 73.3 \pm 3.3%, compared with the control. The data we obtained suggest that increase in the time of impact of equilibration solution on the morule had negative consequences on the development of embryo.

When determining the optimal period of equilibration for blastocysts of cattle, we analyzed the percentage of re-expanded blastocoels and hatching of embryos. We determined some differences in the development of embryos after 10 and 12 min contacts with the equilibration solution, compared with the control (without freezing, Table 2), whereas during 15 min equilibration, the parameters corresponded to the control: percentage of hatched blastocysts accounted for 93.3 \pm 6.7% and 93.3 \pm 3.3%, respectively. Optimal parameters indicate high efficiency of vitrification of cattle blastocysts using the Cryotech kit in the conditions of 15 min exposure to equilibration solution.

Table 2
Influence of time of equilibration of blastocysts on their survival and further development ($x \pm SE$, $n = 3$) when using Cryotech Vitrification Kit 101

Equilibration period, min	Blastocysts with re-expanded blastocoels on the 24 th h after thawing	Hatched blastocysts, %
Control (without freezing)	–	93.3 \pm 6.7 ^a
10	86.7 \pm 8.8 ^a	76.7 \pm 3.3 ^b
12	93.3 \pm 6.7 ^a	83.3 \pm 6.7 ^a
15	96.7 \pm 3.3 ^a	93.3 \pm 3.3 ^a

Note: “–” – impossible to calculate, since re-expansion of the embryo is possible only in the conditions of its freezing; letters indicate significant differences between the groups within the column ($P < 0.05$) according to the results of the Tukey test.

Three minutes decrease in the equilibration period led to 3.4% decrease in the percentage of re-expansion of blastocoels (Fig. 3 and 4) and 10% decrease in hatching, compared with the 15 min equilibration. Ten minutes equilibration was less effective. The percentage of blastocysts with re-expanded blastocoels was 10% lower compared with the group where the equilibration lasted 15 min. At the same time, the percentage of blastocyst hatching in this experimental group was significantly lower than such observed for fresh non-vitrified embryos (76.7 \pm 3.3% against 93.3 \pm 6.7%, respectively). The obtained data suggest decrease in the efficiency of vitrification when shortening the action of equilibration toward blastocyst.

Discussion

In order to effectively introduce the vitrification technology into practice, the protocols used for thawing and transfer of cryoconserved embryos of cattle should be as simple as possible to implement and be efficient at the same time. Currently, slow freezing of embryos is becoming less popular in spite of vitrification. Sunshoff et al. (2011) in their studies indicate that vitrification of *in vitro*-obtained embryo is more effective than the slow freezing, since in the studies, the percentage of expanded blastocysts after thawing equaled 81.1 \pm 20.3% and 79.4 \pm 16.5%, respectively. Gupta et al. (2017) also demonstrated advantages of vitrification, for the percentage of formation of blastocysts after thawing of vitrified morules accounted for 77% compared with 34% after slow freezing.

The vitrification protocol is usually used for human embryos and is now being approved in animal husbandry. Vitrification is characteristic of both increase in rates of freezing and increase in cryoprotectant concentration. However, it has to be noted that most cryoprotectants have a number of negative effects such as toxicity and osmotic damages. Nonetheless, there is no unanimous opinion on toxicity of permeating cryoprotectants. Their toxicity was observed to increase over time with temperature and concentration (Wang et al., 2007; Martínez-Rodero et al., 2021). The toxicity of cryoprotectants is actually considered the single most limiting factor for the development of successful protocols of cryoconservation of cells and the tissues (Szurek & Eroglu, 2011). Therefore, any vitrification protocol must have adequate balance between the production of high level of dehydration, high viscosity and minimal toxicity. The first step of vitrification is the stage of equilibration in solution with relatively low concentration of cryoprotectant, the second – ultrashort exposure (30–90 s) to

vitrification solution with low concentration of cryoprotectant (usually double initial concentration) and the third – dehydration (usually, disaccharides are used, for example sucrose). Equilibration period varies broadly: it can be short (1 min) (Vieira et al., 2007; Sanches et al., 2016), last for 3 min (Gómez et al., 2020; Oliveira et al., 2020) or long (10–15 min) (Morató et al., 2010; Gutnisky et al., 2013).



Fig. 3. Collapsed cavity of cattle blastocysts right after thawing: zona pellucida (a), subzonal space (b), cell mass (c)



Fig. 4. Fully re-expanded cavity of cattle blastocyst on the 24th h after thawing: zona pellucida (a), blastocyst cavity (b), inner cell mass (c), trophoblast (d)

As with the equilibration period, the protocols of commercial kits recommend it being dependent on recovery of embryo form, which could be evaluated by visualization under stereomicroscope. However, in practice, during embryo vitrification at the blastocyst stage, as well as morule, it is often hard to evaluate whether decrease or expansion has occurred, and additionally some blastocysts do not decrease in the vitrification solution. Study of this stage of vitrification is important, for it provides adequate survival of the embryo, hatching of blastocysts and level of nutrition (Martínez-Rodero et al., 2021), because prolonged action of equilibration solution can be harmful to the potential of development of embryo, while insufficient action may influence the permeation of cryoprotectants into

blastomeres. Vitrification of cattle embryos is in the scope of studies by a large number of scientists, who propose different variants of cryoprotectant solutions to produce commercially appropriate results. Therefore, in the conditions of vitrification in TCM-199 solution with addition of HEPES/bicarbonate buffer, 20% calf serum, and also dimethyl sulfoxide and ethylene glycol as cryoprotectant and after thawing in 1 M solution of saccharose and further *in vitro* cultivation, Vajta et al. (1996) determined that 79% of blastocysts re-expanded and 59% hatched. They emphasize that the rates of re-expansion and hatching were different from blastocysts that had been vitrified on the 7th and 8th days (84 and 69% and 70 and 41%, respectively). Souza et al. (2018) consider the combination of 25% ethylene glycol, 25% dimethyl sulfoxide and 1.0 M saccharose optimal for vitrification of cattle blastocysts. It allowed them to obtain 75% of re-expanded blastocoels and 70% hatching. By using the new method of application of hollow fiber vitrification for cryopreservation of morules, Uchikura et al. (2016) observed blastocysts of vitrified embryos forming at rates (66.1–82.5%) similar to those of non-vitrified embryos (74.5–82.5%). By using straw vitrification dilution, Inaba et al. (2011) obtained 97.3% of hatched blastocysts on the 48th day of the cultivation.

Authors of many reports indicate the further development of the embryo as being dependent on the stage of its development during vitrification. Pugh et al. (2000) report equilibration of the embryos for 10 min in 10% ethylene glycol at room temperature, vitrified using one in 40% ethylene glycol, two in 25% ethylene glycol and 25% dimethyl sulfoxide or three in 20% ethylene glycol, 20% dimethyl sulfoxide and 10% 1,3-butanediol cryoprotectants (with or without adding polyvinylpyrrolidone + trehalose), and observed no effect of vitrification solution on survival of embryos after 24 h. However, they observed dependence between the embryo development stage and its survival. Therefore, after 24 h, compacted morules survived in lower amount than blastocytes (24% against 75%, respectively) or hatched after 48 h (15% against 59%, respectively). Our comparison of the effect of equilibration time on embryos at development stages of blastocysts and morules also indicated variability in its influence. Therefore, optimal period of interaction between morules and equilibration solution was 12 min, whereas for blastocysts it was 15 min. The indicated dependence could be explained by the data of Vanderzwalmen et al. (2002), indicating that short equilibration led to low concentration of permeating cryoprotectants inside the blastocoel, which was insufficient to protect blastocysts from the formation of ice crystals inside the cavity of embryos during the freezing stage, thereby decreasing their survival after thawing. However, a smaller morule, as compared with blastocyst (Fig. 1 and 2), has no cavity, and needs therefore less time for its saturation with cryoprotectant.

Kocyigit et al. (2016) report relationship between the ability of blastocysts to re-expand and their diameter. In particular, this parameter for embryos of 100–150 μm diameter accounted for 69.6% and was significantly higher than in other groups (100–150 μm – 52.2% and less than 100 μm – 47.4%). Abdalla et al. (2010) indicate the dependence of cryo-survival on stage of embryo development (expanded blastocyst or complete) and method of its production (intra cytoplasmic sperm injection or co-cultivation with spermatozooids). Therefore, re-expansion of blastocoels of expanded blastocysts obtained by intra cytoplasmic sperm injection (80%) within 24 h was similar to their analogs obtained using extracorporeal insemination (88%). However, compete blastocysts obtained using intra cytoplasmic sperm injection had lower ability to survive the vitrification procedures (61%) than blastocysts obtained using extracorporeal insemination (85%).

Analysis of the described data may suggest that using Cryotec commercial solution for vitrification and thawing is more effective and simpler than schemes proposed by other scientists. In addition, our results of experimental studies are coherent with the data of Gutnisky et al. (2013), who – by using vitrification of blastocysts with Vitrification Kit 101 – achieved 100% survival of *in vitro*-derived cow embryos on the 7th day of the development.

Conclusion

Therefore, the study of vitrification of 4-day morules of cattle produced in *in vitro* conditions using Cryotech Vitrification Kit with 12 min

exposure to equilibration solution revealed the results after thawing which were similar to such for fresh non-vitrified morules. Blastulation of $46.7 \pm 3.3\%$ in the 24th h after thawing, $96.7 \pm 3.3\%$ – in the 48th h and $90.0 \pm 0.0\%$ of hatched blastocysts corresponded to the parameters in intact group – $43.3 \pm 3.3\%$, $96.7 \pm 3.3\%$ and $90.0 \pm 5.7\%$, respectively. The best result of survival of *in vitro*-derived cattle blastocysts (7th day of cultivation) after vitrification using Cryotech Vitrification Kit was seen after equilibration period extended to 15 min. We obtained $96.7 \pm 3.3\%$ of blastocysts that recovered blastocoels after thawing on the 24th day and $93.3 \pm 3.3\%$ hatching, which corresponded to that in the intact group of blastocysts ($93.3 \pm 6.7\%$).

Thus, the obtained results indicate the high efficiency of Cryotech Vitrification Kit, which allowed the hatching of greater amount of embryos during the cultivation after thawing, and survival of vitrified embryos did not differ from the parameters of embryos without vitrification. The presented protocols of vitrification may have important consequences for commercial programs of transplantation of *in vitro* embryos, because they decrease the period that is necessary for vitrification of each certain embryo, and facilitate the use of this technique in production conditions.

References

- Aardema, H., Bertijn, I., van Tol, H., Rijnveld, A., Vernooij, J., Gadella, B. M., & Vos, P. (2022). Fatty acid supplementation during *in vitro* embryo production determines cryosurvival characteristics of bovine blastocysts. *Frontiers in Cell and Developmental Biology*, 10, 837405.
- Abdalla, H., Shimoda, M., Hara, H., Morita, H., Kuwayama, M., Hirabayashi, M., & Hochi, S. (2010). Vitrification of ICSI- and IVF-derived bovine blastocysts by minimum volume cooling procedure: Effect of developmental stage and age. *Theriogenology*, 74(6), 1028–1035.
- Aono, A., Nagatomo, H., Takuma, T., Nonaka, R., Ono, Y., Wada, Y., Abe, Y., Takahashi, M., Watanabe, T., & Kawahara, M. (2013). Dynamics of intracellular phospholipid membrane organization during oocyte maturation and successful vitrification of immature oocytes retrieved by ovum pick-up in cattle. *Theriogenology*, 79(8), 1146–1152.
- Arshad, U., Sagheer, M., González-Silvestry, F. B., Hassan, M., & Sosa, F. (2021). Vitrification improves *in vitro* embryonic survival in *Bos taurus* embryos without increasing pregnancy rate post embryo transfer when compared to slow-freezing: A systematic meta-analysis. *Cryobiology*, 101, 1–11.
- Asgari, V., Hosseini, S. M., Forouzanfar, M., Hajian, M., & Nasr-Esfahani, M. H. (2012). Vitrification of *in vitro* produced bovine embryos: Effect of embryonic block and developmental kinetics. *Cryobiology*, 65(3), 278–283.
- Bó, G., & Mapletoft, R. (2013) Evaluation and classification of bovine embryos. *Animal Reproduction*, 54, 344–348.
- Boland, M. P., Goulding, D., & Roche, J. F. (1991). Alternative gonadotrophins for superovulation in cattle. *Theriogenology*, 35, 5–17.
- Campos-Chillón, L. F., Walker, D. J., de la Torre-Sanchez, J. F., & Seidel Jr., G. E. (2006). *In vitro* assessment of a direct transfer vitrification procedure for bovine embryos. *Theriogenology*, 65(6), 1200–1214.
- Capper, J. L., Cady, R. A., & Bauman, D. E. (2009). The environmental impact of dairy production: 1944 compared with 2007. *Journal of Animal Science*, 87(6), 2160–2167.
- Do, V. H., & Taylor-Robinson, A. W. (2020). Cryopreservation of *in vitro*-produced bovine embryos by vitrification: In pursuit of a simplified, standardized procedure that improves pregnancy rates to promote cattle industry use. *Biotechnology in Animal Husbandry*, 36(3), 251–270.
- Dobrnisky, J. R. (2002). Advancements in cryopreservation of domestic animal embryos. *Theriogenology*, 57(1), 285–302.
- Dochi, O. (2019). Direct transfer of frozen-thawed bovine embryos and its application in cattle reproduction management. *The Journal of Reproduction and Development*, 65(5), 389–396.
- Egger-Danner, C., Cole, J. B., Pryce, J. E., Gengler, N., Heringstad, B., Bradley, A., & Stock, K. F. (2015). Invited review: Overview of new traits and phenotyping strategies in dairy cattle with a focus on functional traits. *Animal*, 9(2), 191–207.
- Estudillo, E., Jiménez, A., Bustamante-Nieves, P. E., Palacios-Reyes, C., Velasco, I., & López-Omelas, A. (2021). Cryopreservation of gametes and embryos and their molecular changes. *International Journal of Molecular Sciences*, 22(19), 10864.
- Ferré, L. B., Kjelland, M. E., Taiyeb, A. M., Campos-Chillon, F., & Ross, P. J. (2020). Recent progress in bovine *in vitro*-derived embryo cryotolerance: Impact of *in vitro* culture systems, advances in cryopreservation and future considerations. *Reproduction in Domestic Animals*, 55(6), 659–676.
- Gerber, P. J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falucci, A., & Tempio, G. (2013). Tackling climate change through livestock – a global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations, Rome.
- Gómez, E., Carrocerá, S., Martín, D., Pérez-Jáñez, J. J., Prendes, J., Prendes, J. M., Vázquez, A., Murillo, A., Gimeno, I., & Muñoz, M. (2020). Efficient one-step direct transfer to recipients of thawed bovine embryos cultured *in vitro* and frozen in chemically defined medium. *Theriogenology*, 146, 39–47.
- Gupta, A., Singh, J., Dufort, I., Robert, C., Dias, F. C. F., & Anzar, M. (2017). Transcriptomic difference in bovine blastocysts following vitrification and slow freezing at morula stage. *PLoS One*, 12(11), e0187268.
- Gutnisky, C., Alvarez, G. M., Cetica, P. D., & Dalvit, G. C. (2013). Evaluation of the Cryotech Vitrification Kit for bovine embryos. *Cryobiology*, 67(3), 391–393.
- Hasler, J. F. (2014). Forty years of embryo transfer in cattle: A review focusing on the journal *Theriogenology*, the growth of the industry in North America, and personal reminiscences. *Theriogenology*, 81(1), 152–169.
- Hasler, J. F., Bilby, C. R., Collier, R. J., Denham, S. C., & Lucy, M. C. (2003). Effect of recombinant bovine somatotropin on superovulatory response and recipient pregnancy rates in a commercial embryo transfer program. *Theriogenology*, 59, 1919–1928.
- Inaba, Y., Aikawa, Y., Hirai, T., Hashiyada, Y., Yamanouchi, T., Misumi, K., Oh-take, M., Somfai, T., Kobayashi, S., Saito, N., Matoba, S., Konishi, K., & Imai, K. (2011). In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. *Journal of Reproduction and Development*, 57(4), 437–443.
- Inaba, Y., Miyashita, S., Somfai, T., Geshi, M., Matoba, S., Dochi, O., & Nagai, T. (2016). Cryopreservation method affects DNA fragmentation in trophectoderm and the speed of re-expansion in bovine blastocysts. *Cryobiology*, 72(2), 86–92.
- Janati Idrissi, S., Le Bourhis, D., Lefevre, A., Emond, P., Le Berre, L., Desnoës, O., Joly, T., Buff, S., Maillard, V., Schibler, L., Salvetti, P., & Elis, S. (2021). Lipid profile of bovine grade-1 blastocysts produced either *in vivo* or *in vitro* before and after slow freezing process. *Scientific Reports*, 11(1), 11618.
- Jennifer, A., Hernandez, G., & Craig, A. G. (2013). Role of reproductive biotechnologies in enhancing food security and sustainability. *Animal Frontiers*, 3(3), 14–19.
- Juanpanich, T., Suttiropattana, T., Takayama, M., Liang, Y., Dochi, O., Pampai, R., & Imai, K. (2018). Survival and developmental competence of bovine embryos at different developmental stages and separated blastomeres after vitrification in different solutions. *Animal Science Journal*, 89(1), 42–51.
- Kocyigit, A., & Cevik, M. (2016). Correlation between the cryosurvival, cell number and diameter in bovine *in vitro* produced embryos. *Cryobiology*, 73(2), 203–208.
- Liebemann, J., & Tucker, M. J. (2006). Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertility and Sterility*, 86(1), 20–26.
- Lövendahl, P., Difford, G. F., Li, B., Chagunda, M. G. G., Huhtanen, P., Lidauer, M. H., Lassen, J., & Lund, P. (2018). Review: Selecting for improved feed efficiency and reduced methane emissions in dairy cattle. *Animal*, 12(s2), s336–s349.
- Mahmoudzadeh, A. R., Van Soom, A., Ysebaert, M. T., & de Kruif, A. (1994). Comparison of two-step vitrification versus controlled freezing on survival of *in vitro* produced cattle embryos. *Theriogenology*, 42(8), 1389–1397.
- Martínez-Rodero, I., García-Martínez, T., Ordóñez-León, E. A., Vendrell-Flotats, M., Olegario Hidalgo, C., Esmoris, J., Mendibil, X., Azcarate, S., López-Béjar, M., Yeste, M., & Mogas, T. A. (2021). Shorter equilibration period improves post-warming outcomes after vitrification and in straw dilution of *in vitro*-produced bovine embryos. *Biology*, 10(2), 142.
- Moore, S. G., & Hasler, J. F. (2017). A 100-year review: Reproductive technologies in dairy science. *Journal of Dairy Science*, 100, 10314–10331.
- Morató, R., Izquierdo, D., Paramio, M. T., & Mogas, T. (2010). Survival and apoptosis rates after vitrification in cryotop devices of *in vitro*-produced calf and cow blastocysts at different developmental stages. *Reproduction, Fertility and Development*, 22, 1141–1147.
- Mucci, N., Aller, J., Kaiser, G. G., Hozbor, F., Cabodevila, J., & Alberio, R. H. (2006). Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. *Theriogenology*, 65(8), 1551–1562.
- Najafzadeh, V., Bojsen-Møller Secher, J., Pihl, M., Aerenlund, A., Jørgensen, N., Jensen, K. K., Jensen, M. T., Fenner, M. F., Strøbech, L., & Hyttel, P. (2021). Vitrification yields higher cryo-survival rate than slow freezing in biopsied bovine *in vitro* produced blastocysts. *Theriogenology*, 171, 44–54.
- Nedambale, T. L., Dinnyés, A., Groen, W., Dobrnisky, J. R., Tian, X. C., & Yang, X. (2004). Comparison on *in vitro* fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. *Theriogenology*, 62, 437–449.
- Oliveira, C. S., da Silva Feuchard, V. L., de Freitas, C., da Silva Rosa, P. M., dos Camargo, A. J. R., & Saraiva, N. Z. (2020). In-straw warming protocol improves survival of vitrified embryos and allows direct transfer in cattle. *Cryobiology*, 97, 222–225.
- Parrish, J. J., Susko-Parrish, J. L., Leibfried-Rutledge, M. L., Critser, E. S., Eyestone, W. H., & First, N. L. (1986). Bovine *in vitro* fertilization with frozen-thawed semen. *Theriogenology*, 25(4), 591–600.

- Petrescu-Mag, R. M., Bumy, P., Banatean-Dunea, I., & Petrescu, D. C. (2022). How climate change science is reflected in people's minds: A cross-country study on people's perceptions of climate change. *International Journal of Environmental Research and Public Health*, 19(7), 4280.
- Pugh, P. A., Tervit, H. R., & Niemann, H. (2000). Effects of vitrification medium composition on the survival of bovine *in vitro* produced embryos, following in straw-dilution, *in vitro* and *in vivo* following transfer. *Animal Reproduction Science*, 58, 9–22.
- Rall, W. F., & Fahy, G. M. (1985). Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. *Nature*, 313(6003), 573–575.
- Raney, T., Skoet, J., & Steinfeld, H. (2009). *The state of food and agriculture: Livestock in the balance*. Food and Agriculture Organization of the United Nations, Rome.
- Rienzi, L., Gracia, C., Maggiulli, R., LaBarbera, A. R., Kaser, D. J., Ubaldi, F. M., Vanderpoel, S., & Racowsky, C. (2017). Oocyte, embryo and blastocyst cryopreservation in ART: Systematic review and meta-analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Human Reproduction Update*, 23(2), 139–155.
- Rocha, J. C., Passalia, F., Matos, F. D., Maserati Jr, M. P., Alves, M. F., Almeida, T. G., Cardoso, B. L., Basso, A. C., & Nogueira, M. F. (2016). Methods for assessing the quality of mammalian embryos: How far we are from the gold standard? *JBRA Assisted Reproduction*, 20(3), 150–158.
- Sanches, B. V., Lunardelli, P. A., Tannura, J. H., Cardoso, B. L., Colombo Pereira, M. H., Gaitkoski, D., Basso, A. C., Arnold, D. R., & Seneda, M. M. (2016). A new direct transfer protocol for cryopreserved IVF embryos. *Theriogenology*, 85, 1147–1151.
- Shirazi, A., Nazari, H., Ahmadi, E., Heidari, B., & Shams-Esfandabadi, N. (2009). Effect of culture system on survival rate of vitrified bovine embryos produced *in vitro*. *Cryobiology*, 59(3), 285–290.
- Siberski-Cooper, C. J., & Koltes, J. E. (2021). Opportunities to harness high-throughput and novel sensing phenotypes to improve feed efficiency in dairy cattle. *Animals*, 12(1), 15.
- Sommerfeld, V., & Niemann, H. (1999). Cryopreservation of bovine *in vitro* produced embryos using ethylene glycol in controlled freezing or vitrification. *Cryobiology*, 38(2), 95–105.
- Souza, J. F., Oliveira, C. M., Lienou, L. L., Cavalcante, T. V., Alexandrino, E., Santos, R. R., Rodrigues, A. P. R., Campello, C. C., Figueiredo, J. R., & Dias, F. E. F. (2018). Vitrification of bovine embryos followed by *in vitro* hatching and expansion. *Zygote*, 26(1), 99–103.
- Stinshoff, H., Wilkening, S., Hanstedt, A., Brüning, K., & Wrenzycki, C. (2011). Cryopreservation affects the quality of *in vitro* produced bovine embryos at the molecular level. *Theriogenology*, 76(8), 1433–1441.
- Szurek, E. A., & Eroglu, A. (2011). Comparison and avoidance of toxicity of penetrating cryoprotectants. *PLoS One*, 6(11), e27604.
- Uchikura, A., Matsunari, H., Nakano, K., Hatae, S., & Nagashima, H. (2016). Application of hollow fiber vitrification for cryopreservation of bovine early cleavage stage embryos and porcine morula-blastomeres. *Journal of Reproduction and Development*, 62(2), 219–223.
- Vajta, G., Holm, P., Greve, T., & Callesen, H. (1996). Overall efficiency of *in vitro* embryo production and vitrification in cattle. *Theriogenology*, 45(3), 683–689.
- Vajta, G., Holm, P., Kuwayama, M., Booth, P. J., Jacobsen, H., Greve, T., & Callesen, H. (1998). Open Pulled Straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. *Molecular Reproduction and Development*, 51(1), 53–58.
- Valente, R. S., Marsico, T. V., & Sudano, M. J. (2022). Basic and applied features in the cryopreservation progress of bovine embryos. *Animal Reproduction Science*, 239, 106970.
- Vanderzwalmen, P., Bertin, G., Debauche, C., Standaert, V., van Roosendaal, E., Vandervorst, M., Bollen, N., Zech, H., Mukaida, T., Takahashi, K., & Schoysman, R. (2002). Births after vitrification at morula and blastocyst stages: Effect of artificial reduction of the blastocoelic cavity before vitrification. *Human Reproduction*, 17(3), 744–751.
- Viana, J. (2020). 2019 statistics of embryo production and transfer in domestic farm animals: Divergent trends for IVD and IVP embryos. *Embryo Technology Newsletter*, 38(4), 1–15.
- Vieira, A. D., Forell, F., Feltrin, C., & Rodrigues, J. L. (2007). In-straw cryoprotectant dilution of IVP bovine blastocysts vitrified in hand-pulled glass micropipettes. *Animal Reproduction Science*, 99, 377–383.
- Wang, X., Hua, T. C., Sun, D. W., Liu, B., Yang, G., & Cao, Y. (2007). Cryopreservation of tissue-engineered dermal replacement in Me₂SO: Toxicity study and effects of concentration and cooling rates on cell viability. *Cryobiology*, 55(1), 60–65.
- Wilmut, I., & Rowson, L. E. A. (1973). Experiments on the low-temperature preservation of cow embryos. *Veterinary Rehabilitation*, 92, 686–690.