485. Modelling of direct-transfer vitrification of bovine embryos

J. Lamy[1*], A. de Wit[1], F. Fotinga-Meiring[2], E. Mullaart[2], L. Zak[3], E. Knol[3], E. Ellen[1] and H. Woelders[1]

[1]Wageningen Livestock Research, Wageningen University & Research, P.O. Box 338, 6700 AH Wageningen, the Netherlands; [2]CRV, Wassenaarweg 20, 6843 NW, Arnhem, the Netherlands; [3]Topigs Norsvin, Research Center, Schoenaker 6, 6641 SZ, Beuningen, the Netherlands; julie.lamy@wur.nl

Abstract

Development of an effective direct transfer vitrification procedure for biopsied *in vitro* derived embryos is necessary for large scale use in the field and increased genetic gain in the cattle industry. One possible reason for insufficient results to date may be the swelling of embryos following warming/transfer. We used mathematical modelling of the volume changes of embryos during and following vitrification protocols, to study the effect of different temperatures and concentrations of cryoprotective agents (CPAs) and non-permeant solutes. The simulations showed no effect of temperature on the level of post-warming swelling at the assumed parameterizations. However, adapted vitrification media with lower concentrations of CPAs and higher non-permeant solutes led to clearly lower swelling after direct transfer, and could potentially be less toxic for the embryos, but also led to a stronger shrinking during vitrification. The alternative media and protocol seem promising, but empirical experiments are needed to validate this approach.

Introduction

Assisted reproductive techniques, such as *in vitro* fertilization and embryo transfer, are used in the cattle industry to decrease the generation interval and increase the genetic gain per generation (Crowe *et al.,* 2021*).* For instance, embryos may be biopsied to obtain a genomic estimated breeding value of each embryo. Embryos can then be frozen and stored in Liquid Nitrogen (LN_2). The use of frozen embryos increased over the past decade and in 2018, a total of 1.1 million of bovine embryos (*in vivo* derived or *in vitro*) were transferred of which 38.2% were frozen (Viana *et al.,* 2019). Direct transfer (DT) cryopreservation protocols allow a straw with an embryo to be thawed and used at the farm without laborious and complicated steps. This is very important for large scale use in the field as it does not require on-site equipment or expertise. Slow-freezing (SF) followed by direct transfer is currently possible and used for bovine embryos (Dochi *et al.,* 2019). Slow freezing at relatively low concentrations of cryoprotective agents (CPAs) allows formation of extracellular ice, which prevents intracellular ice formation. However, survival rates after SF of biopsied *in vitro* produced bovine embryos are lower than with vitrification (Najafzadeh *et al.,* 2021). In contrast, vitrification is fast and doesn't allow any ice formation, but requires high concentrations of cryoprotective agents and high cooling and warming rates. Current vitrification protocols result in good pregnancy rates, but do not allow direct transfer, hence they require one to three washing steps in the field (Ferré *et al.,* 2020). Therefore, DT vitrification methods (no washing steps) need to be developed. Addition of CPAs prior to vitrification, and removal of CPAs after vitrification can lead to stresses caused by massive volume changes, extremely high intracellular concentrations of CPAs and cytoplasmic salts, dehydration of the cellular cytoplasm, and excessive swelling of the cells upon removal of CPAs. We use mathematical modelling to describe transmembrane fluxes of water and CPAs following addition of CPAs prior to vitrification, and removal of CPAs after vitrification. Mathematical modelling allows us to 'look inside cells' as we can accurately calculate not only the volume changes of the cells but also the intra- and extracellular concentrations of water and all solutes at every point in time. The objectives of the present study were to use the mathematical modelling to explore possibilities to mitigate risks of swelling following direct transfer of vitrified bovine embryos. More precisely, we investigated the effect of different concentrations of permeant and non-permeant CPAs in the vitrification solution and of different temperatures after vitrification-warming on post-DT swelling.

R.F. Veerkamp and Y. de Haas (eds)

DOI: 10.3920/978-90-8686-940-4_485, © J. Lamy *et al.* 2022

Materials & methods

General mathematical model. The modeling of osmotic events was done using two parameter (2P) description of the transmembrane fluxes of water and CPA (Kleinhans, 1998). In this description, fluxes of water and CPA are proportional to the transmembrane difference of the chemical potential (ΔM) for water and for CPA, respectively. A full description will be detailed elsewhere (Woelders *et al.,* in preparation). It was assumed that the 'isotonic' (physiological) osmolality is 0.29 Osm/kg of water. Further, in the modelling, the embryo was considered as a single 'cell' that is confined by a single semipermeable outer cell membrane. This assumption is appropriate as the applied membrane permeability coefficients are 'empirical' phenomenological coefficients obtained by fitting actual empirical osmotic volume excursions of embryos. Assumed values for the permeability coefficients and other embryo characteristics were based on reported values for bovine blastocysts, morula's, and earlier embryo stages for various different CPAs (Woelders *et al.*, 2007; Jin *et al.*, 2011). Thus, it was assumed that embryos at isotonic conditions have volume and surface area of a sphere with a diameter (excluding the zona pellucida) of 120 µm, that the embryos surface area remains constant, and that the initial (isotonic) aqueous fraction is 0.8. Further it was assumed that the hydraulic conductivity, or L_p (at 25 °C, in the presence of EG and DMSO) is 1.0 µm/min/atm, that the membrane permeability for permeant CPAs (P_s) is equal for EG and DMSO, and has a value (at 25 °C) of 20 µm/min, and that the activation energy (E_a) of L_p is 6.5 kcal/mol and E_a of P_s is 7.3 kcal/mol. L_p and P_s at other temperatures were calculated as described in Woelders and Chaveiro, 2004.

Simulations. 'Standard' vitrification featured incubation in 'vitrification solution 1' (VS1; base medium with 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO)) for 5 minutes, followed by incubation in 'vitrification solution 2' (VS2; base medium with 15% EG, 15% DMSO, 0.69M of sucrose, and 10 mg/ml Ficoll 400,000) for 1 minute, all at room temperature (RT; 22 °C). The osmolality of VS2 was 12 Osmol/kg of water. Direct transfer was then simulated as an immediate return to base medium (isotonic). Subsequently, similar simulations were run using different temperatures during 'direct transfer', as illustrated in Figure 1.

Then, we investigated the effect of the final amount of (permeant) CPAs taken up by the embryos on the level of embryo swelling after 'direct transfer'. This was done by using variations of VS1 and VS2, with reduced concentrations of the permeant CPAs, combined with elevated concentrations of the non-permeant solute sucrose in VS2, with in all cases a final osmolality of VS2 of 12 Osm./kg water, i.e. identical to that of the 'standard' vitrification protocol. In all VS1 and VS2 media the vol% of EG was equal to that of DMSO. Embryos were incubated in VS1 at RT for 10 min, allowing them to approach equilibrium. The molar concentrations of all base medium solutes was kept constant in all VS1 variants. For reasons explained in

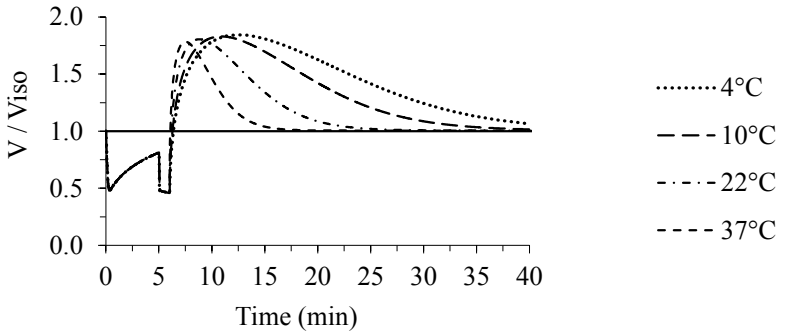

Figure 1. Predicted swelling of bovine embryos in a 'standard' vitrification protocol using different temperatures after warming/transfer. V/Viso is the embryo volume relative to the initial volume (Viso).

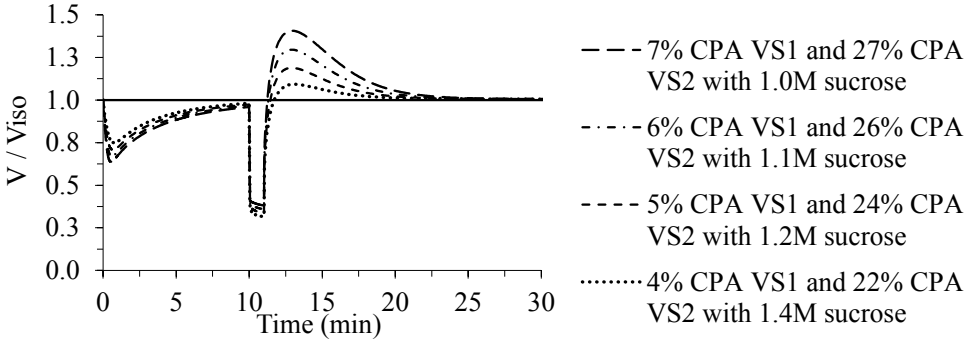

Figure 2. Predicted effect of VS1/VS2 with lower [CPA], with increased sucrose in VS2, all at room temperature. V/Viso is the embryo volume relative to the initial volume (Viso).

the discussion, in all VS1-VS2 combinations the ratio of permeant and non-permeant solutes in VS2 was made equal to that of VS1. VS1 and VS2 concentrations of CPA and sucrose are detailed in Figure 2.

Discussion

In this study, we used a mathematical model to predict volume changes of bovine embryos in different vitrification protocols, with a focus on swelling of the embryos following warming. As is well known, embryos first shrink rapidly after addition of CPA, due to osmotically driven rapid efflux of water, then reswell due to the continued entry of CPA. In two-step vitrification protocols, embryos in VS1 are usually allowed to reswell, i.e. to take up sufficient amounts of CPA approaching equilibrium. In a second (shorter) step in VS2, which has a much higher CPA concentration, the embryos merely shrink due to a rapid efflux of water, which concentrates all solutes inside the embryo. Transfer of embryos after vitrification/warming to a medium with less or with no CPAs is known to lead to strong swelling of embryos above their isotonic volume. Our simulations show that in the 'standard' protocol direct transfer to isotonic medium leads to swelling of the embryos to 1.8 times their initial volume (Figure 1). This represents the 'worst-case scenario', as we assumed immediate and complete mixing with isotonic medium. In reality, embryos placed together with a small volume of VS2 into the uterus will be progressively exposed to isotonic conditions, so swelling will be less. However, the level of swelling is considered to be potentially harmful. We have investigated whether the level of swelling depends on the temperature of the medium to which the embryos are exposed after warming. The rapidity of the swell and shrink cycle strongly depended on temperature, but temperature had only little effect on the peak level of swelling. The latter is due to the fact that at the assumed values of E_a of L_p and E_a of P_s, the rates of water flux and CPA flux had a similar dependence on temperature. If E_a of P_s were substantially higher, peak swelling could be much lower, but one would need to assume an unrealistically high value, four times higher than the value we used, to bring peak swelling down to 1.2 × Viso (data not shown).

Another approach we followed was to reduce the CPA intake of the embryos. In fact, this is what is achieved in protocols with shortened incubation in VS1, with the 'standard' (high) concentration of CPA (Ortiz-Escribano *et al.*, 2018). Here, we used a longer incubation (full equilibration) with a strongly reduced CPA concentration in VS1. The full equilibration allowed us to have control over the amount of CPA taken up by the embryos. The low concentrations of CPA in VS1 (4-7 vol.%, or 0.64-1.1 mol/l) were in the range of what is commonly used in slow freezing protocols, which is considered harmless for embryos during a 10 minute incubation. In VS2, we used elevated sucrose and lower CPA concentrations compared with the 'standard' VS2 such that the ratio of permeant and non-permeant solutes in VS2 was equal to that in VS1. This

ensures that there was no or little net influx or efflux of CPA during the short incubation in VS2. However, a rapid efflux of water was observed in VS2, which brought the intracellular osmolality to the level of the extracellular osmolality (of VS2) within a few seconds. This approach was shown to be highly effective in reducing the peak level of swelling of embryos following direct transfer (to 1.2-1.4× Viso). However, the lower concentration of CPA in VS1 led to stronger shrinking in VS2 (just prior to vitrification), with V/Viso of 0.32-0.38, compared with 0.46 in the standard protocol. The stronger shrinking may pose larger mechanical stress on the embryos, but at the same time, the lower concentrations of CPA used in VS1 and VS2 could be less toxic for the embryo. Empirical experiments are needed to show if these adapted media and protocols will result in adequate embryo survival and whether or not these sufficiently reduce post-warming swelling to allow direct transfer. This study was financially supported by the Dutch Ministry of Economic Affairs (TKI Agri and Food project).

References

Dochi O. (2019) J. Reprod. Dev. 65(5):389-396. https://doi.org/10.1262/jrd.2019-025

Jin B., Kawai Y., Hara T,, Takeda S., Seki S., *et al* (2011) Biol. Reprod. 89(4):87. https://doi.org/10.1095/biolreprod.112.107250

Kleinhans F.W. (1998) Cryobiology 37:271-289. https://doi.org/10.1006/cryo.1998.2135

Najafzadeh V., Secher J.B.M., Pihl M., Ærenlund A., Jørgensen N. *et al.*, (2021) Theriogenology 171:44-54. https://doi.org/10.1016/j.theriogenology.2021.04.020

Ortiz-Escribano N., Bogado Pascottini O., Woelders H., Vandenberghe L., De Schauwer C., (2018) Equine Vet J. 50(3):391-397. https://doi.org/10.1111/evj.12747

Ponsart C., Le Bourhis D., Knijn H., Guyader-Joly C., Otter T. *et al.*, (2013) Reprod. Fertil. Dev.26(1):12-21. https://doi.org/10.1071/rd13328

Viana J. (2020) Embryo Technology Newsletter, v. 38, n.4

Woelders H., Chaveiro A. (2004) Cryobiology 49(3):258-271. https://doi.org/10.1016/j.cryobiol.2004.09.001

Woelders H., Mullaart E., Merton S. (2007) Proc. of 23rd Annual Meeting A.E.T.E. Alghero, Italy.